Humans naturally acquire cross-specific anti-glycan antibodies

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“Wir setzten den Fuß in die Luft

- und sie trug”

Hilde Domin

Abbreviations:

7-AAD	7-Aminoactinomycin D
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AID	Activation-induced cytidine deaminase
APC	Allophycocyanin
AUC	Area under curve
ASC	Antibody-secreting cell
BCR	B cell receptor
bp	Basepairs
BSA	Bovine serum albumin
BV	Brilliant violet
CD	cluster of differentiation
CD40L	Cluster of differentiation 40 ligand
cDNA	complementary DNA
CDR	Complementary-determining region
CSR	Class-switch recombinantion
Cy	Cyanin
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTE	Dithioerythritol
DTT	Dithiothreitol
Ec	*Eschecheria coli*
EDTA	Ethylendiamintetraacetat
ELISA	Enzyme-linked immunosorbent assay
ESBL	Enhanced-spectrum β-lactamase
Fab	Variable region
FACS	Fluorescence-activated cell sorting

Fc	Constant region
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
FWR	Framework region
GC	Germinal center
HD	Healthy donor
HEK	Human Embryonic Kidney
HRP	Horseradish-Peroxidase
Ig	Immunoglobulin
ILF	Isolated lymphoid follicle
Kp	*Klebsiella pneumoniae*
LB	Lysogeny Broth
LP	Lamina propria
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MS	microbiota staining
NMR	Nuclear magnet resonance
OD	Optical density
OTU	Operational taxonomic unit
PAGE	Polyacrylamide gel electrophoresis
PB	Peripheral blood
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PP	Peyer's patch
rDNA	ribosomal DNA
RHP	Random Hexamer Primer
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium

SA	Streptavidin
Sc	*Saccharomyces cerevisae*
SDS	Sodiumdodecyl sulfate
SHM	Somatic hypermutation
Sp	*Streptococcus pneumoniae*
STD	Saturation transfer difference
TBS	Triss-buffered saline
TB	Terrific broth
TD	T cell-dependent
TFH	Follicular helper T cell
TI	T cell-independent
WHO	World Health Organization

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Abstract

Bacterial glycan antigens are highly diverse in composition and linkage. Antibodies against glycan antigens can protect against bacterial infection and are important in maintaining homeostasis between the host and its microbiome. However, glycan antigens typically elicit B cell responses that have impaired long-term memory formation and are comprised of low-affine antibodies with low specificity. In this work, the use of biotinylated Lipopolysaccharide O-antigens of the opportunistic pathogen *Klebsiella pneumonia* allowed to identify anti-O-antigen B cells in the peripheral memory and intestinal effector B cell pool in healthy humans. Single B cell Immunoglobulin gene sequencing, antibody cloning, and recombinant expression reveal that, under natural circumstances, humans acquire affinity-matured antibodies against defined *Kp* glycan antigens. Despite their O-serotype-specificity, the antibodies bind to other structurally similar *Kp* O-serotypes and taxonomically distinct non-*Kp* microbes. The findings show that humans, under natural exposure, acquire affinity-matured cross-specific anti-glycan antibodies and provide a mechanistic way how the humoral immune system could adapt to the large microbial glycan diversity present in nature. Further, the antibodies identified in this work might be beneficial in treatment of nosocomial *Kp* infections.

Abstract (German)

Bakterielle Glykanantigene sind hoch-divers in ihrer Komposition und Verbindung. Antikörper gegen Glykanantigene können vor bakteriellen Infektionen schützen und sind wichtig um die Homöostase zwischen dem Wirt und seinem Mikrobiom aufrecht zu halten. Typischerweise lösen Glykanantigene jedoch Antikörperantworten aus, die sich durch ein vermindertes B Zell-Gedächtnis und niedrig-affine Antikörper mit geringer Spezifität auszeichnen. In dieser Arbeit konnten, mithilfe von biotinylierten Lipopolysaccharide O-Antigenen des opportunistisch-pathogenen Bakteriums *Klebsiella pneumoniae* (*Kp*), O-Antigen-spezifische B Zellen innerhalb peripherer Gedächtnis- und intestinaler Effektor-B Zellen identifiziert werden. Durch Einzel-Zell Immunoglobulin-Sequenzierung und Klonierung bzw. rekombinanter Expression von Antikörpern dieser Zellen wird gezeigt, dass, unter natürlichen Umständen, affinitätsgereifte Antikörper gegen definierte *Kp* Glykanantigene erzeugt werden. Diese Antikörper binden nicht nur *Kp* O-Serotyp-spezifisch sondern auch spezifisch an strukturell ähnliche *Kp* O-Antigene und taxonomisch unterschiedliche Mikroorganismen. Die Ergebnisse zeigen, dass Menschen, bei natürlicher Besiedlung, kreuz-spezifische Antikörper gegen Glykanantigene erzeugen und deuten auf einen Mechanismus hin, wie das humorale Immunsystem auf die Glykandiversität des Mikrobioms reagieren und sich anpassen kann. Weiterhin könnten die hier identifizierten Antikörper nützlich für die Behandlung von nosokomiellen *Kp* Infektionen sein.

1. Introduction

1.1 *Klebsiella pneumoniae*

Klebsiella pneumoniae (*Kp*) belong to the family of *Enterobacteriaceae* and most frequently colonize the human intestinal tract as part of the natural flora.[1] It is estimated that 5-38% of humans are naturally colonized.[1] However, *Kp* has become one of the most frequently isolated gram-negative nosocomial pathogens.[2,3] It mainly infects vulnerable patients such as newborns and immune-compromised patients, where it predominantly causes urinary tract infection, pneumonia or subsequently bacterimia.[4] *Kp* infections mainly appear as hospital outbreaks and are associated with a high mortality rate.[5] In addition to this, a major problem concerning *Kp* is the increasing emergence of multi-drug resistant strains. Especially enhanced-spectrum β-lactamase (ESBL) producing strains are frequently isolated from patients.[2] These strains leave few possible treatment options because of their resistance to carbapenems, an antibiotic of last resort treating *enterobacteriaceae* infections.[5] To date neither a vaccination against *Kp* nor a treatment against multi-drug resistant *Kp* infections is available. Therefore, the development of alternative strategies to directly target multi-drug resistant *Kp* infections, especially ESBL-producing isolates, and to reduce their global prevalence by introducing effective vaccination are major goals in fighting *Kp* infections.[7]

1.1.1 *Klebsiella pneumoniae* O-antigen

Kp are encapsulated gram-negative bacteria. The capsule is a main determinant of virulence by reducing phagocytosis and mediating resistance to complement-mediated lysis.[8,9] It is composed of two polysaccharides, K-antigen and Lipopolysaccharide (LPS). *Kp* K-antigens are highly diverse as more than 77 different so-called K-serotypes have been described.[10] LPS, the other capsule compound, consists of three parts, a lipid moiety so-called lipid A, a core oligosaccharide component, termed common part, and repetitive units of an additional polysaccharide chain, referred to as O-antigen. Lipid A

is widely conserved among gram-negative bacteria and anchors the LPS molecule to the outer membrane. The attached core oligosaccharide is located proximal to the bacterial membrane and is conserved among the *Klebsiellae* genus.[11] In contrast to K-antigen, O-antigen shows limited serotype diversity among *Klebsiellae* and to date seven distinct O-serotypes plus three additional variants have been identified. Favorably for a suggested vaccine candidate, only four O-serotypes, namely O1, O2, O3 and O5, account for the vast majority of clinical isolates (Fig.1A).[11,12] However, recent epidemiological data on the O-serotype distribution of nosocomial *Kp* infections is missing and three O-serotype variants have been newly discovered since last reports (J. Lukasiewicz, unpublished).[13] Additionally, *Kp* O-antigens share similar linear structures between different O-serotypes. O1 and O2 O-antigen share a Galactan I subunit which was recently identified to be modifiable to Galactan III (Fig1B).[13] Furthermore, O3 and O5, despite being mannose homopolymers, share an identical trimannose, which is also present in the O3 LPS variants O3a and O3b (Fig.1C) (J. Lukasiewicz, unpublished).

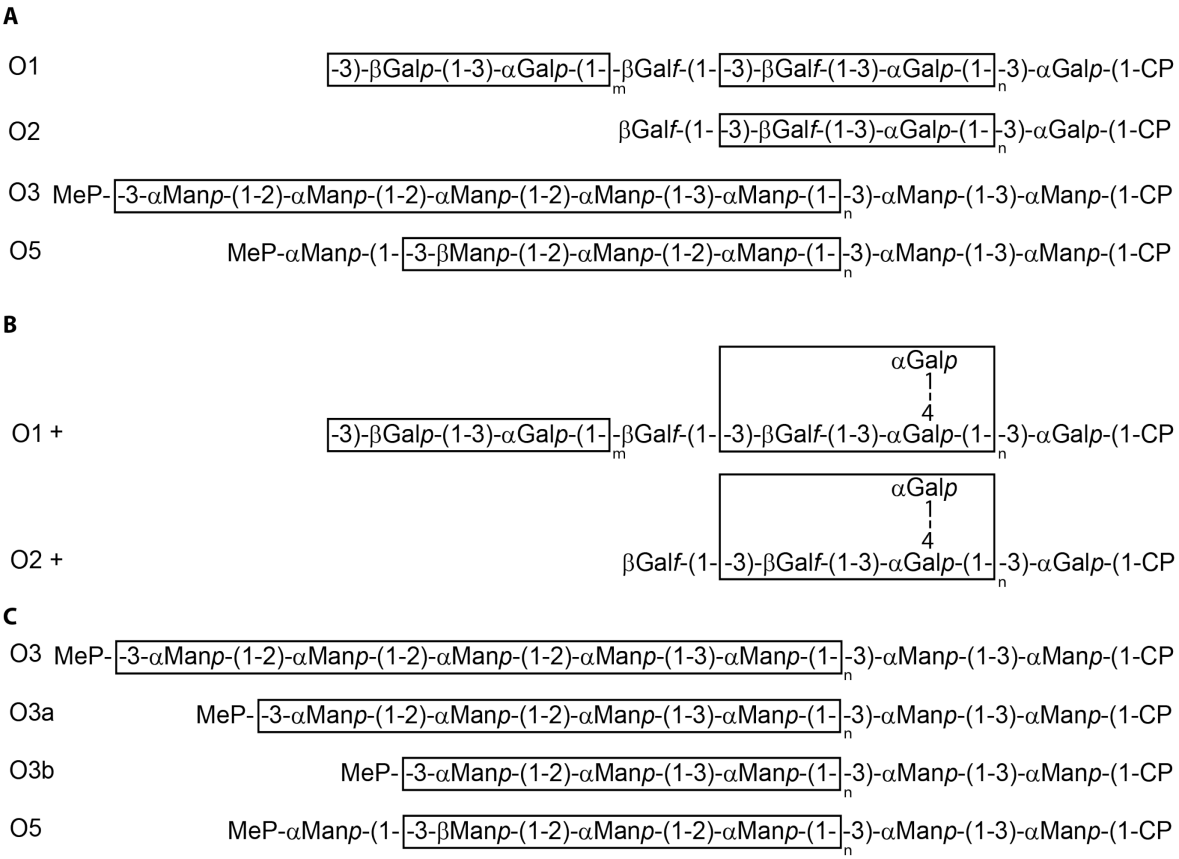


Figure 1: Linear chemical structure of *Klebsiella pneumoniae* O-antigens from (A) the four predominantly isolated clinical isolates, (B) Galactan-III variants O1+ and O2+ and (C) mannan-based O3, O3 variants O3a and O3b, and O5 O-serotypes. Repetitive units are depicted in squares; CP: common part.

1.1.2 Vaccination against *Klebsiella pneumoniae*

Successful vaccination strategies against bacteria rely on the formation of immunological memory where the induction of protective antibodies plays a crucial role in conferring immunity.[14] The capsule of *Kp* is suggested to be one of the major vaccine targets for protective antibodies preventing *Kp* infection.[10] However, vaccination attempts using a 24-valent K-antigen vaccine of the most abundant K-serotypes, despite eliciting protective antibody responses in patients, did not exceed phase 1 trials due to low K-serotype coverage and the lack of long-term protection.[10,15,16] Given its low serotype diversity O-antigen is another promising vaccine candidate, especially because a murine antibody that targets O-antigen has been shown to be beneficial in experimental *Kp* infections.[17] However, the problem of O-antigens is that glycan antigens typically poorly induce B cell memory formation and antibodies of low affinity and specificity.

1.2 B cell memory

By definition, memory B cells are an antigen-experienced population bearing pathogen-specific B cell receptors (BCRs) and persisting long term within the entire B cell pool.[18] There are two major B cell subsets which confer immunological memory: long-lived plasma cells and memory B cells. The former are terminally-differentiated cells residing in bone marrow niches for decades, contributing specific antibodies to the serum.[19] The latter are circulating cells in the periphery and rapidly activated upon antigen re-encounter. Upon pathogen re-encounter, they can differentiate into antibody-secreting cells (ASCs) and quickly contribute specific antibodies to the serum.[20] In humans, a specific, but not exclusive, molecular marker for human memory B cells is the elevated surface expression of cluster of differentiation 27 (CD27).[21,22] A further hallmark of

memory B cells, even more specific than CD27, is the production of somatically mutated high affinity B cell receptors.[23] Thus, obtaining Immunoglobulin (antibody-encoding) gene information and reactivity data about antibodies are essential to determine the status of a given B cell population. However, it is unclear if humans mount B cell memory against *Kp* O-antigens.

1.3 The B cell receptor

BCRs are membrane-embedded proteins belonging to the Immunoglobulin (Ig) superfamily. A BCR is composed of two identical IgHeavy (IgH) and IgLight (IgL: Igκ or Igλ) chains, covalently linked by disulfide bridges (Fig.2). They are exclusively expressed by

B cells. Alternative splicing of the trans-membrane domain leads to secretion of BCR into the body fluids and the BCR is thus referred to as antibody. While the membrane-embedded BCR mediates B cell-intrinsic signaling, the secreted antibody acts on B cell-extrinsic antibody-mediated effector functions. Despite this difference, BCR and antibody share their structural motifs and functional domains. Antibodies can be subdivided into a variable (Fab) and a constant (Fc) region. The variable region non-covalently binds antigen and consists of two antigen-binding sites, each built by one IgH and IgL chain variable region. The IgH variable region is encoded by *IGHV*, *IGHD* and *IGHJ* gene segments, whereas the Igκ and Igλ variable regions are encoded in the *IGKV* and *IGKJ* and *IGLV* and *IGLJ* gene segments, respectively. Structural studies of antibodies indicate that the antigen-binding site is shaped by the complementary-determining regions (CDRs) 1-3 of the Ig gene, whereas the structural integrity of the Fab is maintained by the frame-work regions (FWRs) of the Ig gene. In contrast, the Fc domain, which is defined only by the IgH chain, modulates the effector function of the molecule. In humans, there are nine different constant regions ordered by sequence homology into five different constant classes containing one to four subclasses: IgA (1-2), IgD, IgE, IgG (1-4) and IgM. Importantly, B cells can switch their antibody constant region by class-switch recombination (CSR) and therefore can modulate an ongoing immune response towards pro-inflammatory or anti-inflammatory conditions.

Exemplified, IgG1 antibody is the most pro-inflammatory subclass in humans, whereas IgG2 can dampen an ongoing immune response towards a pathogen.[24,25]

The Ig class of an antibody also modulates its binding nature by increasing the valency and, hence, binding avidity. By definition, avidity is the binding strength of an antibody-antigen complex achieved by multiple binding sites each with a certain affinity. Increasing binding avidity is achieved by multimerization of single antibody molecules by the Joining chain.[26] IgM is mainly secreted as pentamer, IgA predominantly as dimer whereas IgD, IgE and IgG are secreted as monomers. Given the two antigen-binding sites per antibody molecule, pentavalent IgM possesses dodecavalent binding sites and thus higher avidity than a bivalent IgG antibody antigen interaction with the same affinity.

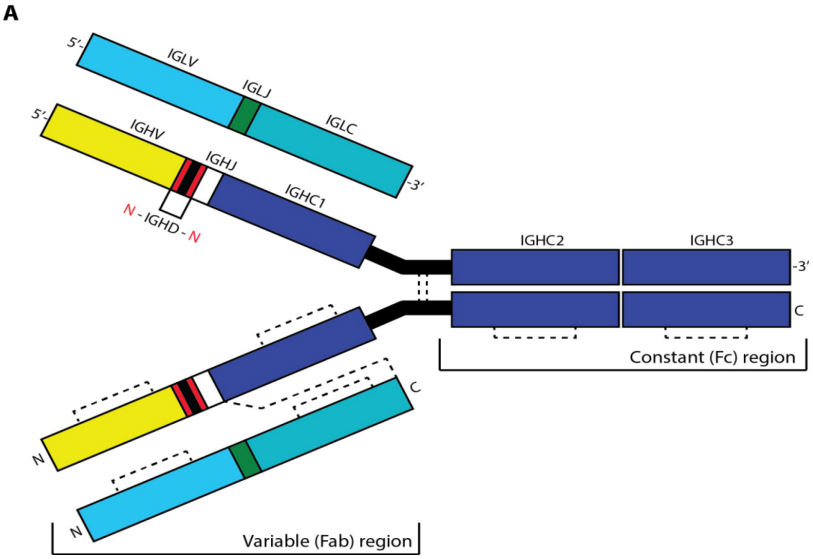


Figure2: Schematic representation of an IgG1 antibody molecule. Antibody molecules can be divided in the constant (Fc) and variable (Fab) region encoded by the IgHeavy and IgLight chain. Top shows the arrangement of the Ig genes and the location of N nucleotide addition (red) in the IgHeavy chain. Dashed lines describe disulfide bridges responsible for heterodimer formation (bottom).

1.4 Generation of diversity in the antibody repertoire

An exceeding majority of B cells in the human B cell pool is assumed to express a unique BCR.[27] Thus, humoral immunity is equipped to react to an almost infinite number of structurally diverse antigens. There are three distinct mechanisms how B cell repertoire

diversity is generated. First, during B cell development in the bone marrow, B cells randomly recombine V(D)J segments to functional IgH and IgL chain, a process referred to as V(D)J somatic recombination. The process involves the Recombination-activating gene (RAG)-mediated random recombination of *IGHV*, *IGHD* and *IGHJ* gene segments to a functional IgH variable gene and *IGKV* and *IGKJ* or *IGLV* and *IGLJ* to a functional IgK or IgL variable genes, respectively (Fig.3A)[28,29]. Further, during this process diversity is increased by insertion of non-templated (N) nucleotides within each Ig gene joint.[30] Second, B cells pair a given IgH chain with either an IgK or IgL chain. Third, a B cell with a given BCR can introduce somatic hypermutation (SHM) within its Ig genes to randomly generate high-affinity BCR variants. This process is referred to as affinity maturation and occurs in micro-anatomical structures, so-called germinal centers, in secondary lymphoid organs.[31]

1.5 T-cell dependent antibody responses

Mature naïve (antigen-inexperienced) B cells circulate in the blood and through the secondary lymphoid organs such as the lymph nodes and the spleen. Upon antigen encounter, antigen-specific B cells internalize antigen, get activated and home to the T cell / B cell border in the secondary lymphoid follicles.[32] Subsequently, they get fully activated by interaction with cognate $CD4^+$ helper T cells via MHC class II:peptide complexes and further crucial co-receptors, most importantly CD40/CD40L interaction.[33] As a result, a proportion of antigen-reactive B cells proliferates and differentiates into extra-follicular antibody-secreting cells (ASCs), which can undergo initial CSR.[34] Thus, a first wave of antibodies is released into the body fluids. Another portion of B cells migrate into the follicle to seed germinal centers (GCs), where diversification by SHM of Ig genes occurs.[35] It was shown that both pathways are not restricted to recently activated naïve B cells and that formerly acquired memory B cells can as well be able to seed germinal centers.[36]

In parallel to GC B cells, cognate $CD4^+$ T cells differentiate into T follicular helper (TFH) cells, a specialized T cell subset within the GC. In the GC reaction specific B cells proliferate, can undergo CSR, and acquire random point mutations in their IgH and IgL variable regions by induction of the enzyme Activation-induced cytidine deaminase

(AID).[37] It is noteworthy that CSR and SHM are both AID-dependent mechanism, but it has been observed that one can occur independent of the other.[38–40] The GC model assumes that B cells with different BCR variants compete for limited amount of antigen deposited onto follicular dendritic cells and in parallel for TFH cell help. Randomly generated high-affinity BCR variants receive more antigen, subsequently, stronger survival stimuli from TFHs and are therefore predominantly selected.[41] Continuously during the GC reaction, high-affinity B cells exit the GC and differentiate into antibody-secreting cells (ASC's) or memory B cells. It is assumed that relatively lower affinity clones preferentially enter the memory B cell pool.[42] However, high-affinity antibody variants can often be found in the memory B cell population.[43,44]

1.6 T cell independent antibody responses

In contrast to antibody responses against proteins, glycan antigens such as O-antigen cannot be presented via MHC class II molecules and do not elicit T cell help (Fig.3).[45] TI antibody responses can be classified in two subtypes (TI-1 and TI-2), depending on the nature of their B cell-activating stimulus. TI-1 antigens activate B cells independently of the BCR. For example, lipid A is a potent polyclonal B cell activator via Toll-like receptor 4 (TLR4).[46] In contrast, repetitive glycan antigens elicit TI-2 antibody responses. Here, multiple BCR engagements on the surface of the cell lead to activation of the B cell.[47] However, to form a robust TI antibody response, B cells need to be activated via BCR stimulation and co-stimulatory signals such as TLR4.[48]

Despite the general capability of B cells to be activated and to differentiate into ASCs, lack of T cell help results in particular characteristics of TI antibody responses. First, affinity maturation in GCs is impaired.[47] As a consequence, anti-glycan antibodies derive from extra-follicular antibody responses, display low SHM and suggestively consist of low-affine antibodies.[49,50] However, examples of somatically mutated monoclonal antibodies targeting bacterial glycan antigens have been described in humans.[51–53]

Second, vaccination attempts using pure TI antigens in humans have suggested that B cell memory is not or inefficiently formed in TI responses.[54] Thus, TI responses fail to

induce robust recall responses and long-term protection.[55] However, Obukhanych *et al* showed that a TI-2 antigen generally can elicit B cell memory which can be adoptively transferred to littermates.[56] In humans, asplenic, splenectomized, and individuals with common variable immunodeficiency, who suffer from increased susceptibility to infections by encapsulated bacteria such as *Streptococcus pneumoniae (Sp)*, led to the identification of splenic MZ B cells and their circulating counterparts (IgM memory cells) as essential in protecting against bacterial infections.[57] In line with this, IgM memory has been found to secrete anti-glycan antibodies.[58] Nonetheless, in humans, the formation of B cell memory against *Kp* under natural circumstances and especially the quality of antibodies against bacterial polysaccharides remains elusive.

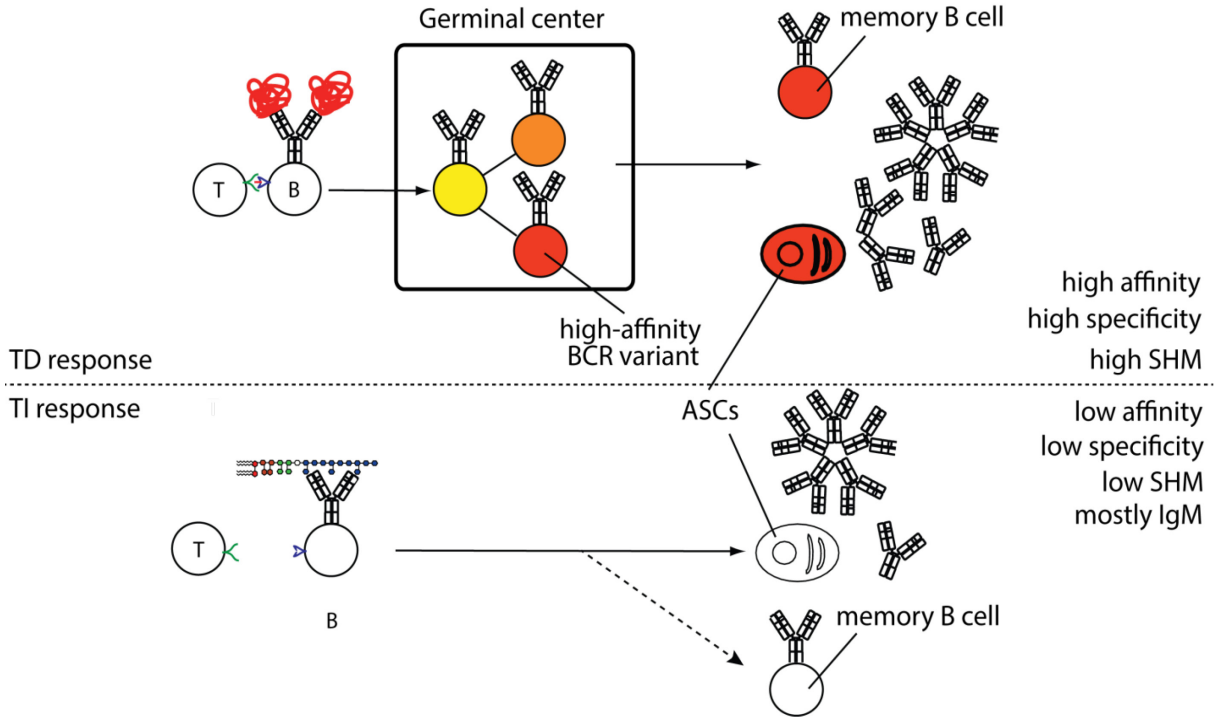


Figure 3: T cell-dependency of antibody responses. In a T cell-dependent antibody response, interaction of a B cell with its cognate T cell results in selection and generation of high-affinity BCR variants in the GC reaction. These high-affinity variants differentiate into memory B cell or ASCs (top). TI antibody responses cannot induce T cell help and thus result in low affinity antibodies with low specificity, low SHM, and do preferentially not undergo CSR.

1.7 Intestinal humoral immunity

The intestine displays the biggest surface in the human body. The intestinal immune system is exposed to a diverse microbiome, providing a huge diversity of protein and glycan antigens with bacterial, archeal, viral, fungal and food origin. Intestinal antibody responses play an important role in maintaining homeostasis between the host and the microbiome, e.g. by preventing invasion, neutralizing antigens, regulating bacterial virulence and in antigen-sampling from the intestinal lumen.[59–63] Intestinal antibodies derive from lamina propria (LP) plasmablasts that populate the intestinal LP, the effector site of intestinal B cell responses. To cross the tightly sealed epithelium layer, antibodies are shuttled from the basolateral site of the gut epithelium and secreted into the lumen by the action of the polymeric Ig receptor.[64] The inductive sites of these antibody responses are two micro-anatomical structures, referred to as Peyer's patches and isolated lymphoid follicles (ILFs).[65] Both provide a unique cytokine milieu, which favors induction of CSR to IgA. Thus, IgA is the main Ig subclass found in the gut, however, around 6-18% and 3-4% percent of LP plasmablasts in human gut express IgM and IgG under steady state conditions, respectively.[66] Peyer's patches are micro-anatomical structures that are mainly found in the ileum of the small intestine. They harbor microbiota-induced GCs which are constitutively present and active, as they sample antigen from the intestinal lumen by specialized microfold cells.[67] Affinity maturation in these PP GCs was shown to be the main driver in generating antigen-specific IgA antibodies in the human intestine and the vast majority of LP plasmablasts are supposed to derive from PP GCs.[68] Apart from that, LP plasmablasts can also be generated in ILF's.[69] In contrast to Peyer's patches, ILFs lack T cell zones and are therefore supposed to be predominantly involved in TI antibody responses.[70]

In general, antibody responses in the human intestine are suggested to be comprised of two layers of antibodies, “classical” and “innate” (also termed “primitive”) IgA (Fig4).[71] “Classical” IgA is generated in TD antibody responses in GCs. Therefore, these antibodies underwent affinity maturation and are supposed to show specific antigen binding. On the other hand, “innate” IgA are antibodies which were generated in TI antibody responses. These antibodies are suggested to either non-specifically (poly-reactive) coat the microbiota or target evolutionary conserved pattern on the

microbiota.[72] Controversially, BCR repertoires from human LP plasmablasts revealed that basically all antibodies present in human LP are somatically mutated and the majority of cells expressed epitope-specific antibodies, including a monoclonal antibody binding to *Escherichia coli (Ec)* LPS.[51,73] Thus, it has been demonstrated that humans possess glycan-binding antibodies in the intestinal lamina propria, however, the specificity of these antibodies has not been determined yet.

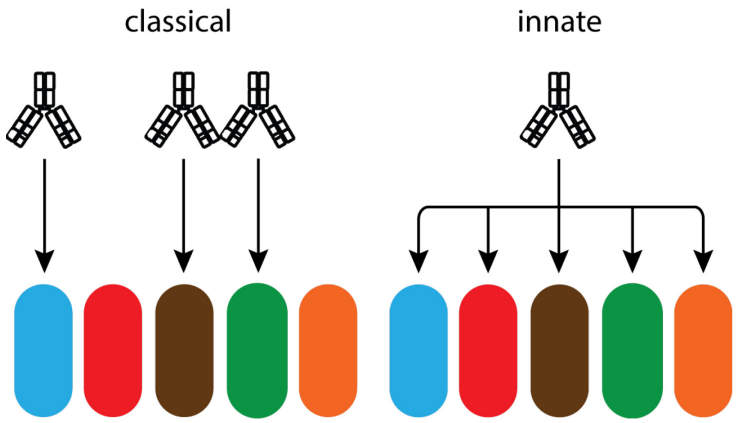


Figure 4: Binding model of antibodies to intestinal microbes. Antibodies (for simplification shown here as monomers) can either be classical, which were generated in TD responses and bind specifically (left) or innate, binding un-specifically (poly-reactive) to all microbes (right).

2. Objectives

The **main aim** of this work was the characterization of the human humoral immune response to *Kp* O-antigen under natural circumstances.

The **specific aims** were:

i) to determine if humans mount peripheral memory and intestinal effector B cells against *Kp* using LPS O-antigen as a model glycan antigen.

ii) to describe the molecular characteristics of the human peripheral memory and intestinal effector anti-O-antigen B cell Ig gene repertoire.

iii) to determine if O-antigen-binding B cells express affinity-matured antibodies.

iv) to elucidate the specificity of O-antigen-binding antibodies.

3. Methods

3.1 Donors

All human specimens obtained in this study were taken after written consent and approval by local ethics committees of the medical universities in Heidelberg ("B Zell Antwort gegen kommensale Darmbakterien bzw. molekulare und funktionale Charakterisierung des humanen intestinalen Antikörper Repertoires"; "B-Zell-Antwort auf Klebsiella pneumoniae"), Leipzig ("B Zell Anwort gegen kommensale Darmbakterien", 011-13-28012013) and Berlin ("B Zell Antwort gegen kommensal Darmbakterien", EA1/257/12; " B-Zell-Antwort gegen Klebsiella pneumoniae", EA1-147-13). Patient data are summarized in Supplementary Table 4.

3.2 Lipopolysaccharide fractions and biotinylation of O-antigen

All Lipopolysaccharide (LPS) fractions were prepared and obtained from Jolanta Lukasiewicz, Ludwik Hirszfeld Institute of Experimental Therapy, Wroclaw, Poland (J. Lukasiewicz, unpublished). Briefly, *Kp* were cultured in 10l LB medium (37 °C, 200 rpm, 5 L/min gas flow). Subsequently, bacteria were killed by addition of 0.5 % Phenol for 2 h at 60 °C, centrifuged, washed with distilled water and freeze-dried. LPS was isolated by the hot phenol/water extraction, purified by dialysis and glass-wool filtration followed by ultracentrifugation as described in Lukasiewicz *et al.*[74] O-polysaccharides were obtained by mild acid hydrolysis (1,5 % acetic acid, 20 min, 100 °C) and subsequently ultra-centrifuged for 6 h with 105.000 g at 4 °C to remove capsular antigens. The supernatant was freeze-dried and fractionated by gel filtration using a Bio-Gel P-10 (200-400 mesh) as previously described.[75] Obtained fractions were controlled by ^{1}H nuclear magnet resonance (NMR) spectroscopy.

Biotinylation of O-antigen was performed using LPS from O1, O2-, O2+ and O3 O-serotypes from strain #063 (O1:K2), #079, Kp26 and O3:K-, respectively (J. Lukasiewicz, unpublished). Structures of biotinylated O-antigen were obtained by

Jolanta Lukasiewicz, Ludwik Hirszfeld Institute of Experimental Therapy, Wroclaw, Poland.

3.3 Isolation of peripheral blood mononuclear cells

Freshly drawn human peripheral blood was diluted 1:1 with RPMI medium (Gibco™, Thermo Fisher Scientific) at room-temperature and slowly added onto 15 ml Ficoll (GE Healthcare) in a 50ml centrifuge tube (Sarstedt). The cells were spun for 40min at room-temperature and 400 g with the lowest acceleration and no break. Cells residing at the water/Ficoll interface were isolated using a Pasteur pipet and resuspended in a minimum of 25 ml RPMI at room-temperature in a 50 ml centrifuge tube. All subsequent steps were performed at 4 °C and on ice. Cells were centrifuged at 400 g for 10 min. The supernatant was discarded and the cells were washed in 10 ml ice-cold RPMI. The cells were counterstained using Trypan Blue (Gibco™, Thermo Fisher Scientific), counted using a Thoma chamber and subsequently centrifuged at 400 g for 10 min. The supernatant was discarded and cells were further processed for flow cytometry or immediately frozen following the freezing protocol.

3.4 Isolation of LP lymphocytes

LP plasmablasts were directly isolated from fresh surgical samples. All cells were isolated from healthy mucosa having at least 3cm distance from the tumor or inflamed area. The source of the tissue is specified in Table 1. Lamina mucosa and propria were dissected from lamina muscularis using forceps and scalpel. The tissue was extensively washed in PBS+ (1x PBS (Gibco™, Thermo Fisher Scientific), 2 % heat-inactivated FCS (Invitrogen), 1x Antimycotic/Antibiotic (Gibco™, Thermo Fisher Scientific) at room-temperature. The tissue was placed onto a petri-dish placed into its lid filled with water and frozen overnight at -20°C. The tissue was cut into 3-5mm pieces and remaining connective tissue (white; forms droplets when removed from solution) was removed as extensively as possible. The tissue was transferred to a 50 ml centrifuge tube (Sarstedt)

and washed 3-times with 1 x PBS+ and subsequently incubated 2 x 15 min with PBS containing 1mM DTE in a 250 ml bottle placed in a water bath at 37 °C to remove residual mucus. Subsequently the tissue was washed 3-times with 1 x PBS containing 0.5 mM EDTA, followed by 30 min incubation with 1 x PBS containing 0.5 mM EDTA at 37 °C as described above to remove the epithelium. After washing with 1 x PBS+ the tissue was digested using 1x PBS+ containing 0.2 % (w/v) DnaseI and 0.5 % (w/v) Collagenase D (both Roche) for 1 h under constant stirring at 37 °C. LP plasmablasts were isolated by a discontinuous Percoll gradient (40 %/70 % diluted in 1 x PBS). To better discriminate the 40 %/ 70 % interface Phenol Red was added to the 70 % dilution (1:1000; Gibco™). 15 ml of each dilution were added into a 50 ml centrifuge tube and 20 ml cell suspension was slowly added onto the top of the gradient. After centrifugation, LP lymphocytes were isolated from the 40 %/70 % interface by using a Pasteur pipet and added into a minimum of 25 ml RPMI into a new centrifuge tube. All subsequent steps were performed at 4 °C and on ice. Cells were centrifuged at 400 g for 10 min. The supernatant was discarded and the cells were washed in 10 ml ice-cold RPMI. The cells were counted using a Thoma chamber using a Trypan Blue counterstain (Gibco™) and subsequently centrifuged at 400 g for 10 min. The supernatant was discarded and cells were further processed for flow cytometry or immediately frozen following the freezing protocol.

3.5 Freezing of mammalian cells

Cells were counted and diluted with heat-inactivated FCS (Gibco™, Thermo Fisher Scientific) to reach a concentration of $1x10^7$ cells/ ml. Heat-inactivated FCS containing 20 % (v/v) sterile DMSO suitable for cell culture (Sigma) was freshly prepared and 500 µl were added to 1.8 ml cryotubes (ThermoFisher). 500µl cell suspension was added to reach a final concentration of $5x10^6$ cells/ ml and the vials were frozen at -80 °C using a Coolcell (Biocision)

3.6 Cell staining for flow cytometry

Flow cytometry cell stainings were performed in 1.5 ml tubes using 1x PBS containing 2 % heat-inactivated FCS (FACS buffer) or Horizon stain buffer (BD) if more than one Brilliant violet dye was used. $5x10^6$ Cells/ ml were stained in 50 µl staining mix for 30 min at 4 °C in the dark using the following antibodies:

Mouse anti-human CD19-APC-H7 (BD), Mouse anti-human CD27-PE (BD), Mouse anti-human CD27-BV605 (BD), Mouse anti-human IgG BV510 (BD), Mouse anti-human IgG V450 (BD), Mouse anti-human IgA-PE (Miltenyi), Goat anti-human IgA-FITC (Life technologies), Mouse anti-human CD45-VioGreen (Miltenyi), and Mouse anti-human CD11b-PE-Cy7 (eBioscience). Dead cells were excluded by 7-AAD (Life technologies). Biotinylated *Kp* O-antigen was used at a final concentration of 20 µg/ml and detected with 0.5 µg/ml Streptavidin-Alexa647 (1:2000 of a 1 mg/ml stock solution).

Using biotinylated O1 and O3 O-antigen in the same flow cytometry analysis was achieved by pre-incubating biotinylated O1 and O3 O-antigen fractions with Streptavidin-Alexa647 (Life technologies) and Streptavidin-BV786 (BD) in a Biotin:Streptavidin molar ratio of 1:1 for 1 h at 4 ^{0}C in the dark, respectively. Subsequently, the conjugates were dialyzed overnight in the dark against 10 l of ice-cold PBS using a 20 k Mw cut-off dialysis unit (Thermo Scientific).

Then, 1 ml FACS buffer was added and the cells were spun for 5 min at 500 g at 4 °C. The supernatant was discarded and the cells were resuspended in 1ml FACS buffer and centrifuged again. If a secondary staining step was necessary the pellet was resuspended in 50 µl staining mix and cells were incubated 30 min at 4 °C in the dark. For washing 1 ml of FACS buffer was added before the centrifugation step. Supernatant was discarded and cells were resuspended in 1 ml FACS buffer prior to centrifugation. After supernatant removal the cells were diluted using FACS buffer (250-1000 µl) and filtered into a tube with a meshed cap (Falcon™, BD) before analysis or sorting.

3.7 Single cell sorting

O-antigen binding memory B cells were identified as single, 7-AAD$^-$, CD19$^+$, CD27$^+$, O-antigen$^+$ cells. LP plasmablasts were isolated as single, 7-AAD$^-$, CD19$^+$, CD45$^+$, O-antigen$^+$. Single cells were sorted on Aria II instruments (BD) into 384-well plates (4titude) containing 2 µl of freshly thawed sort mix (Table 1).

Reagent	Concentration	Volume per well [µl]
water	-	1.4813
PBS	[10x]	0.0500
DTT	[100 mM]	0.1000
NP-40	[10 %]	0.1375
RHP	[300 ng/µl]	0.1375
RNAsin	[40 U/µl]	0.0938

Table 1: Sort mix for single B cell sorting

After single cell sorting the plates were immediately frozen on dry ice and stored at -80 °C or directly processed.

3.8 Ig gene amplification by reverse transcription and nested PCR

Full length Ig genes of single human B cells were obtained by the method described by Tiller *et al* modified by Murugan *et al*. [76,77] Full cDNA of single-cell sorted B cells was synthesized by reverse transcription using random hexamer primers. Plates were heated 1 min to 68 °C to remove secondary and tertiary RNA structures. Subsequently, 2 µl of reverse transcription (RT) mix were added.

Reagent	Concentration	Volume per well [µl]
water	-	0.6375
RT-Buffer	[5x]	0.8000
DTT	[100 mM]	0.3000
dNTPs	[25 mM each]	0.1375
RNAsin	[40 U/ul]	0.0563
SuperScript III	[200 U/µl]	0.0688

Table 2: Reverse transcription mix for cDNA synthesis from single B cells

Plates were shortly centrifuged and RT was performed in a 384-well cycler (Eppendorf) at: 5 min. 42 °C, 10 min. 25 °C, 60 min. 50 °C, 5 min. 94 °C. Afterwards, cDNA was diluted by adding 3 µl of Nuclease-free water (Qiagen) and mixed well. After short centrifugation, 1 µl total cDNA was transferred to a primary 384-well PCR plate containing 9µl of primary PCR mix (Table 3). Primers used to amplify the full length Ig genes were previously published and are summarized in Supplementary Table 5. [51,76–78] To increase efficiency of the amplification process two *IGH* locus primary PCRs were performed. The remaining cDNA was stored at -20 °C.

Reagent	Concentration	Volume per well [µl]
water	-	7.5950
buffer	[10x]	1.0000
primer A	[50 µM]	0.1300
primer B	[50 µM]	0.1300
dNTPs	[25 mM each]	0.1000
HotStar Taq	[5 U/µl]	0.0450
cDNA	-	1.0000

Table 3: Primary PCR mix to amplify Ig gene loci from full cDNA of single B cells

After the primary amplification step, 1.5 µl of both *IGH* primary PCRs were pooled into a new 384-well plate and mixed well. Afterwards, 1 µl of the respective *IGH* mix and *IGK* and *IGL* primary PCR product was transferred into 9 µl of secondary PCR mix (Table 4). To increase the specificity of the secondary amplification the primers were designed to bind in a nested fashion.

Reagent	Concentration	Volume per well [µl]
water	-	7.7900
buffer	[10x]	1.0000
primer C	[50 µM]	0.0325
primer D	[50 µM]	0.0325
dNTPs	[25 mM each]	0.1000
HotStar Taq	[5 U/µl]	0.0450
primary PCR	-	1.0000

Table 4: Secondary PCR mix to amplify Ig genes from primary Ig gene PCR by Nested PCR

All PCR were carried out using the safe mode of the 384-well PCR cycler (Eppendorf). PCR protocols for *IGH* or *IGK* and *IGL* are shown in Table 5 and Table 6, respectively.

Step	Duration [min:sec]	Temperature [°C]	
Activation	15:00	94°C	
Denaturation	00:30	94°C	
Annealing	00:30	58°C	X 50 cycles
Elongation	00:55	72°C	
Elongation	10:00	72°C	
Cooling	∞	4°C	

Table 5: Primary and secondary PCR protocol to amplify *IGH* from single B cells

Step	Duration [min:sec]	Temperature [°C]	
Activation	15:00	94°C	
Denaturation	00:30	94°C	
Annealing	00:30	58°C	X 50 cycles
Elongation	00:45	72°C	
Elongation	10:00	72°C	
Cooling	∞	4°C	

Table 6: Primary and secondary PCR protocol to amplify *IGK and IGL* from single B cells

2 µl of the secondary PCR product was loaded onto a 2 % agarose gel and successful amplification was confirmed by ethidium bromide staining of bands at approximately (due to random nucleotide addition in the CDR3 region) *IGH* =450-500 bp, *IGK* = 500 bp and *IGL* = 500 bp. Sequence information was obtained by Sanger sequencing using the

primer RMX2-A for *IGH*, Ck494 for *IGK* and hCl-040 for *IGL* products. Sequences showing a high mutational load in FWR1 were additionally sequenced using the 3' primer included in the respective secondary PCR to increase the resolution of the region.

3.9 Ig gene cloning

In order to perform antibody reactivity measurements the fully human antibodies were recombinantly produced using the method of Tiller *et al* with small modifications.[76] For antibody production, the *IGH* and the corresponding *IGK or IGL* gene were first cloned into human Igγ1 and Igκ or Igλ expression vectors, respectively. Therefore, the *IGH* and the corresponding *IGK* and/or *IGL* genes were specifically amplified by PCR using V-segment and J-segment specific primers (Supplementary Table 6). For downstream cloning, the primers contained an AgeI and an additional SalI, BsiWI or XhoI restriction site for the amplified *IGH*, *IGK or IGL* segment, respectively (Table 7 and Supplementary Table 6). The PCR cycler protocols used to amplify the Ig genes are shown in Table 5 and 6.

Reagent	Concentration *IGH* / *IGK*	Concentration *IGL*	Volume per well [µl]
water	-	-	28.42
buffer	[10x]	[10x]	4.00
primer V	[3.3 µM]	[50 µM]	2.00
primer J	[3.3 µM]	[50 µM]	2.00
dNTPs	[25 mM each]	[25 mM each]	0.40
HotStar Taq	[5 U/µl]	[5 U/µl]	0.18
primary PCR	-	-	3.00

Table 7: PCR mix to specifically amplify *IGH* and *IGK or IGL* gene loci from primary PCR

2 µl of the specific PCR product was loaded onto a 2 % agarose gel and successful amplification was confirmed by ethidium bromide staining of bands at approximately *IGH* =400, *IGK* = 350bp and *IGL* = 400bp.

The Ig gene DNA fragments were purified using purification columns (Macherey & Nagel) according to the manufacturer's instructions. In brief, DNA fragments bigger than 400 bp were retarded on a silica resin column, residual primers and proteins were removed by washing and the DNA fragment was eluted using 75 µl nuclease-free water.

30.6 µl of the purified PCR Ig gene was mixed with 3.4 µl of the respective buffer (NEB) and mixed with 6 µl of the digestion mix containing the appropriate restriction enzymes (NEB) (Table 8).

Reagent	*IGH* **Volume per well [µl]**	*IGK* **Volume per well [µl]**	*IGL* **Volume per well [µl]**
water	5.30	4.95	5.30
CS buffer [10x]	0.60	-	0.60
NEB 1 buffer	-	0.60	-
[10x]	-	0.40 [100x]	-
BSA [100x]	0.05 AgeI [20 U/µl]	0.05 AgeI [20 U/µl]	0.05 AgeI [20 U/µl]
Rest. enzyme 1	0.05 SalI [20 U/µl]	-	0.05 XhoI [20 U/µl]
Rest. enzyme 2			

Table 8: Digestion mix for cloning of *IGH, IGK* and *IGL* genes

All digestion mixes were incubated 1.5 h at 37 °C. The Igκ digestion mix was additionally digested with BsiWI for 1.5 h at 55 °C. Therefore, a mix of 1.68 µl nuclease-free water, 0.2 µl nebuffer1, 0.1 µl BsiWI and 0.02 µl 100x BSA was added to the reaction and mixed well. To remove the restriction enzymes as well as the overhang fragments the digested DNA fragments were purified as described above and eluted with 50 µl nuclease-free water. Afterwards the purified and digested Specific PCR Ig gene fragments were ligated into human Igγ1 and Igκ or Igλ expression vectors containing the respective human Ig constant region (Supplementary Figure 3). The Igγ1 expression vector was equipped with the secretory splice variant of the Ig constant, enabling the secretion of antibody into the cell culture supernatant. Therefore, 8 µl of the purified digested PCR product was mixed with 1 µl 10x ligation buffer (NEB), 0.5 µl purified digested vector [50 ng/µl], and 0.5 µl T4 DNA Ligase [400 U/µl] (NEB) and incubated for 1 h at room-temperature or at 16 °C overnight.

In order to amplify successfully ligated expression vectors, the vectors were transformed into *Ec* (DH10B). Thus, 10 µl of chemically-competent bacteria were mixed with 3 µl of ligation mix and left on ice for 30 min. Afterwards, the bacteria were transformed by heat-shock at 37 °C for 2 min and left on ice for additional 2 min. To amplify successfully transformed bacteria 100 µl pre-warmed LB medium (Sigma) was

added and the bacteria were incubated for 1 h at 37 °C and 650 rpm on a heat-block (Eppendorf). To select positive clones the whole solution were plated on pre-warmed LB agar plates containing 100 µg/ml Ampicillin and incubated for a minimum of 16 h at 37 °C.

Insertion of the Ig gene was confirmed by PCR using a primer upstream of the leader sequence (Absense) and one located in the corresponding Igγ1, Igκ or Igλ constant region (IgGinternal, Cκ494 or hCl-057, respectively) (Supplementary Table 6). Therefore successfully transformed and resistance-selected colonies of *IGH, IGK and IGL* were picked from the plate using a 10 µl pipet tip, streaked onto a new LB plate containing Ampicillin and subsequently dissolved in Insert-check PCR mix (Table 9). Insert-check PCR was carried out as described in Table 10.

Reagent	Concentration	Volume per well [µl]
water	-	18.6
buffer	[10x]	2.5
Absense	[50 µM]	0.2
IgGinternal, Cκ494 or hCl-057	[50 µM]	0.2
dNTPs	[1.25 mM each]	2.5
self-made Taq	n/a	1.0

Table 9: Insert-check PCR mix for confirmation of successful insertion if Ig genes into the expression vector

Step	Duration [min:sec]	Temperature [°C]	
Bacterial lysis	5:00	94°C	
Denaturation	00:30	94°C	
Annealing	00:30	58°C	X 27 cycles
Elongation	00:60	72°C	
Elongation	10:00	72°C	
Cooling	∞	4°C	

Table 10: Insert-check PCR protocol

4 µl of the Insert-check PCR product was loaded onto a 2 % agarose gel and successful insertion was confirmed by ethidium bromide staining of bands at approximately IgH=650 bp, Igκ = 700 bp, and Igλ = 590 bp.

To confirm correct insertion into the respective expression vector, 5 µl of the Insert check PCR product were sent for purification and sequencing by Sanger sequencing (Eurofins genomics). First, the obtained sequence was checked for in-frame insertion of the respective Ig gene. Afterwards, the sequence was compared to the secondary PCR product sequence and excluded if PCR-prone additional point mutations in the Insert check PCR sequence were found. If point mutations found in the secondary PCR product were not present in the Insert-check PCR sequence, these mutations were not included into the analysis, due to a high likelihood that these mutations were generated early in the secondary PCR process.

To amplify correctly cloned expression vectors, bacteria bearing the correct plasmid were inoculated into 4 ml TB (Gibco™, Thermo Fisher Scientific) containing 75 µg/ml Ampicillin (Sigma) in 13 ml culture tubes (Sarstedt) and grown minimum 16 h at 37 °C

at 180 rpm. The plasmid DNA was extracted using the Nucleospin Kit (Macherey & Nagel) according to the manufacturer's instructions. In brief, the bacteria were lysed, proteins and genomic DNA were coagulated, and plasmid DNA in the supernatant was retarded on silica resin. Plasmid DNA was eluted using two-times 50 µl nuclease-free water and concentration was measured on a Nanodrop (ThermoScientific). A minimum of 1µg plasmid DNA was subjected to long-term storage at -20 °C using cryo-tubes (Greiner Bio-One).

3.10 Antibody expression

Human antibodies are glycoproteins which require the formation of disulfide bridges and a functional glycosylation machinery for correct folding and function. As bacterial expression systems do not provide both mechanisms, the fully human IgG1 antibodies were produced by Polyethylenimine- (PEI) mediated transfection of adherent and non-adherent human embryonic kidney 293 cells, HEK293T and HEK293F, respectively. [76,79]

3.10.1. Mammalian cell culture

HEK293T were cultured at 37 °C in 5 % CO_2 in 25 ml DMEM GlutaMAX™ (ThermoFisherScientific) media containing 10 % (v/v) heat-inactivated FCS and 1 x Antibiotic/Antimycotic (Gibco™, Thermo Fisher Scientific), whereas HEK293F cells were cultured at 37 °C in 5 % CO_2 in 20 ml Freestyle medium (Gibco™, Thermo Fisher Scientific) at 180 rpm in 50 ml Bioreactors (TPP).

3.10.2. PEI-mediated transfection of HEK293T ATCC No. CRL-11268 cells

The cationic polymer PEI was used for transient gene transfer to HEK293T cells. Therefore, 10–15 µg IgH vector was mixed with equal amount of its corresponding IgL vector and 50 µl/µg total DNA of 150 mM sterile sodium chloride solution was added.

Subsequently, PEI [0.6 mg/ml] was added in a 3:1 (w/w) DNA to PEI ratio. The solution was immediately vortexed for 10 s and incubated at room-temperature for 10 min. In the meantime, plates were washed with 10 ml pre-warmed DMEM GlutaMAX™ to remove residual bovine serum antibodies. Thus, 25 ml pre-warmed expression media (DMEM GlutaMAX™ containing 1x Antibiotic/Antimycotic (Gibco™) and 1x serum-free media supplement Nutridoma (Roche) was added and the cells were incubated at 37 °C in 5% CO_2 until further use. Hence, the transfection mix was added drop-wise to the cells and the cells were incubated for 3.5 days. Subsequently, the antibody secreted into the supernatant was harvested and the cells were again incubated with 25 ml expression media. The supernatants were centrifuged at 4000 g to remove cell debris and transferred into a sterile 50 ml centrifuge tube (Sarstedt).

3.10.3. PEI-mediated transfection of HEK293F cells

HEK293F cells (Thermo Fisher Scientific) were transiently transfected using the cationic polymer PEI (Sigma). 10 ml cells were seeded at 1.5 x 10^6 cells/ml, in Freestyle 293 expression medium (Thermo Fisher Scientific), the day before transfection. After 16 h, the cell number was determined to be 2.5 x 10^6 cells/ml using a Thoma chamber. Thus, 10-15µg IgH vector was mixed with equal amount of its corresponding IgL vector and added to the cell suspension. Cells were incubated for additional 5 min. To transfect the prepared cells PEI [0.6 mg/ml] was added in a 3:1 (w/w) DNA to PEI ratio. After 24 h 10 ml Ex-Cell medium (Gibco™, Thermo Fisher Scientific) containing 4 mM L-Glutamine (Gibco™, Thermo Fisher Scientific) was added to the cells and incubated for 5 days at 37 °C in 5 % CO_2. The supernatants were centrifuged at 4000 g to remove cell debris and transferred into a sterile 50 ml centrifuge tube (Sarstedt).

3.11 Antibody purification

In order to purify the secreted antibody from cell culture supernatant 12.5 µl Protein-G-coupled beads (GE Healthcare) per 10 ml antibody containing supernatant were washed with 50 ml ice-cold sterile 1x PBS pH = 7.4 (Gibco™, Thermo Fisher Scientific) by centrifugation at 4000 g 4 °C for 10 min. The supernatant was carefully removed from the beads and an appropriate volume of 100 µl/ sample was left in the centrifugation tube and added to the antibody supernatants. The mixture was incubated for at least 12 h at 4 °C on a rotator. The beads were harvested by centrifugation at 4000 g 4 °C for 10 min and the supernatant was carefully removed and added into a new sterile 50 ml centrifugation tube if needed. The beads were added onto a chromatography column (Bio-Rad) which was equilibrated with 2 ml of ice-cold PBS. The columns were emptied by gravity-flow or by applying pressure with the thumb. Beads were washed with 1.5 ml ice-cold PBS. Antibody was released from Protein-G into a 1.5 ml tube by a low pH pulse applying 450 µl sterile 0.1 M Glycine pH = 3 for 3 min and the solution was buffered by adding a 1:10 equivalent of a sterile 1 M Tris solution pH = 8. The procedure was repeated using 225 µl Glycine solution and eluted into a second sterile 1.5 ml tube. The pH = 7.4 – 8.0 of the solution was confirmed by adding an appropriate amount onto a small pH indicator strip (Sigma).

3.12 Antibody concentration measurement

Antibody concentrations in purified fractions were measured by Enzyme-linked immunosorbent assay (ELISA). Therefore, a 96-well high-binding plate (Costar) was coated with 50 µl 1:500 dilution of a goat anti-human IgG Fcγ-fragment specific capture antibody (Dianova) for at least 12 h at 4 °C. Thus, the plates were washed 3-times with deionized water and 200 µl blocking buffer (1x PBS, 0.05 % Tween 20 and 1 mM EDTA) was added per well for 1 h. After washing additional 3-times, the plates were incubated with eight 50 µl 1:2.5 serial antibody dilutions in PBS and incubated for 1 h. Two serial

dilutions of human IgG from human plasma (Sigma) starting with 1 µg/ml and 3 µg/ml served as standards. After washing, 50 µl of a 1:1000 HRP-coupled goat anti-human IgG secondary antibody was added for 1 h. After an additional washing step, 100 µl HRP ABTS substrate was added and the amount of bound antibody was detected as the optical density at 405 nm.

3.13 O-antigen ELISA

ELISA was used to measure concentration-dependent antibody binding to O-antigen. In order to immobilize biotinylated O-antigen samples, high-binding 96-well ELISA Plates (Costar) were coated overnight with 50 µl of 1 µg/ml Streptavidin (NEB) in PBS at 4 °C. Subsequently, plates were washed 3-times in PBS before adding 50 µl of either 1 µg/ml or 2 µg/ml of one biotinylated O-antigen of Klebsiella pneumoniae strains O3 O3:K-or O1 #063, O2- Kp26 and O2+ #079, respectively. After incubation for 1 h at room-temperature, plates were washed and incubated with 200 µl 2 % BSA in PBS for 1 h at room-temperature. After washing, 1:4 serial dilutions of recombinant human IgG1 antibodies with a starting concentration of 4 µg/ml were added to the plate for 1 h at room-temperature. After an additional washing step, concentration-dependent binding was detected using 50 µl 1:1000 goat anti-human IgG Fc HRP-coupled (Jackson) secondary antibody diluted in blocking buffer. After washing, 100 µl HRP ABTS substrate was added and antibody binding was detected as optical density at 405 nm.

3.14 Streptavidin ELISA

Binding to Streptavidin was performed as described above for the O-antigen ELISA. The only difference was that instead of diluted biotinylated O-antigen the plate was incubated with the same amount of PBS.

3.15 Insulin ELISA

Non-specific binding to insulin was measured by as described for the O-antigen ELISA. The only difference was that instead Streptavidin a 10 ug/ml solution of recombinant human Insulin (Sigma) was coated overnight, washed and directly blocked using 200 µl 2 % BSA solution.

3.16 LPS Immunoblot

Antibody-binding to full LPS fractions was assessed using Immunoblot. 2 µg LPS/sample of *Kp* O-serotypes was diluted in SDS-containing loading dye (NEB) and heated for 5 min to 95 °C before applied onto an anyKd gradient SDS-PAGE (Bio-Rad). To prevent shrinking of the gel in the subsequent transfer the gel was placed for at least 5 min in transfer buffer. Meanwhile, a nitrocellulose membrane was cut into an appropriate size and activated in Methanol for 1 min, washed in deionized water for 1 min, and equilibrated in transfer buffer for at least 5 min. LPS was transferred by semi-dry Immunoblot for 1 h at 1 mA/cm^2 onto a nitrocellulose membrane (GE Healthcare) and subsequently fixed onto the membrane by complete drying at room-temperature for up to 30min. After re-activation of the membrane by methanol for 1 min and an additional washing step in water for 1 min, the membrane was placed overnight in a 4 % BSA in TBS solution. Following this, the membrane was cut with a scalpel into appropriate pieces and incubated in a 2 µg/ml monoclonal human IgG1 antibody in TBS solution for 1.5 h at room-temperature. Subsequently, the membrane was washed 3-times with TBS for 5 min and incubated with anti-human IgG Fc HRP-coupled secondary antibody 1:10000 in TBS containing 1 % BSA. After washing 3-times with TBS for 5 min, binding was assessed using luminol-based detection (Pierce).

3.17 Antibody binding to microbiota using flow cytometry

The intestine of eight 16-32 weeks-old male C57BL6/J originating pairwise from different cages and housing was opened longitudinally and intestinal bacteria were harvested by vigorously vortexing the tissue with 90 ml of sterile LB-medium. The tissue was removed, 10 ml of sterile DMSO was added, and the samples were frozen at -20 ^{0}C until further use. All subsequent steps were performed at 4 ^{0}C. 500 µl of the bacteria suspension was mixed on ice with 1 ml 1x PBS containing 1 % BSA microbiota staining (MS) buffer and centrifuged for 15 min at 50 g to remove large particles. The supernatant was transferred into a new tube and centrifuged at 8000 g and for 5 min. The pellet was resuspended in 100 µl MS buffer containing 4 µg/ml antibody and incubated for 30 min. 1 ml MS buffer was added and the samples were centrifuged at 8000 g for 5 min. The pellet was washed in 1 ml MS buffer and centrifuged again before adding 100 µl of 1 µg/ml goat anti-human IgG Alexa-647 secondary antibody (Jackson) in MS buffer for 30 min. The washing steps were repeated before the pellet was diluted in at least 2 ml of MS buffer before acquisition or sorting.

IgG-bound populations were sorted as depicted in Fig 11A-C. 1.500 – 100.000 events per antibody were sorted on a BD FACS Aria II into sterile FACS tubes using the purity sort mode. The tube was rinsed with 500 µl sterile PBS, transferred into a new 1.5 ml tube, centrifuged for 5 min at 8000 g, the supernatant was discarded and the samples were frozen at -20 ^{0}C. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions and eluted into 50 µl Nuclease-free water (Qiagen). 11.5 µl genomic DNA was mixed with each 0.5 µl of 341F forward primer (5'-CCTACGGGNGGCWGCAG-3') and 805R reverse primer (5'-GACTACHVGGGTATCTAATCC-3'), and 12.5 µl of peqGOLD Hot Start Mix Y (Peqlab) to amplify the V3-V4 region of the 16S ribosomal DNA (rDNA) by PCR. To combine up to 64 different samples the forward primer was indexed with an 8nt barcode (Supplementary Table 7). To reduce the amplification bias the PCR was carried out in triplicates for: 5min 94^{0}C; 34-times 45s at 94^{0}C, 1min at 55^{0}C, 1min30sec at 72^{0}C; 10min at 72^{0}C. Successful amplification was confirmed and all reactions were pooled,

separated by gel-electrophoresis and the band at ~460 bp was cut and purified using the Nucleospin Gel and PCR Clean-up (Macherey-Nagel) according to the manufacturer's instructions and eluted into 100 µl Nuclease-free water. The 16S rDNA was prepared for Illumina sequencing using PCR-free adaptor ligation and sequenced using the Miseq250bp v2 chemistry by the DKFZ sequencing core facility. To detect cross-contamination an empty FACS tube which underwent the exact same treatment was added to the workflow. Obtained sequences were assembled with pandaseq v2.8 using the pear algorithm and assigned by their barcoded primer to the respective sample allowing for 1.5 mismatches using the QIIME suite.[80,81] Next, the sequences were assigned to operational taxonomic units (OTUs) of 99% sequence similarity using Open reference picking performed by QIIME. A minimum of 12587 sequences per sample was identified and, thus, the detection limit of 1/12587 was added to the OTU abundance of each OTU within each sample. Next, all OTUs showing a higher abundance of 11/12587 in the empty control and low abundant OTUs (<20/12587 in all samples) were deleted. To identify enriched OTUs the relative abundance of each OTU within the sample was divided by the relative abundance within the whole mixture or, as control, the antibody-bound population of the non-polyreactive mature naïve control antibody mGO53.[78]

3.18 Germline reversion

Germline antibodies were produced as IgG1 using Ig gene cloning and antibody expression as described above. Ig germline genes were obtained by gene synthesis at MWG Eurofins Genomics. Noteworthy, the addition of random N-nucleotides in the IgH and IgL CDR3 region during somatic recombination does not allow reversion of this region. Thus, if available, the CDR3 region of the B cell cluster member showing minimal mutational load was generated.

3.19 Ig gene analysis

Human Ig genes were identified using the Ig gene reference database of IMGT Version 1.2.1 embedded into the NCBI Ig Blast. The best matched germline hit was identified. If the query sequence resulted in two identical scores the Ig gene with the smaller gene or allele number was chosen to facilitate cluster identification. Somatic hypermutations (SHM) were counted from the end of the V gene-specific primer-binding region until the end of the *IGHV, IGKV*, or *IGLV* gene. Insertions or deletions regardless of their length were counted as one SHM. Ig isotype subclasses were determined by sequence homology to published human constant regions (http://www.ensembl.org).

3.20 Statistics and Bioinformatics

Statistics were performed using Prism 6 (Graphpad) and R version 3.2.2 (The R foundation) (* $P<0.05$, ** $P<0.005$, *** $P<0.0005$). Statistical tests are indicated in the figure legends. Single cell sort fluorescence index data was recorded using BD FACSDIVA 7 V8.0.1 software and extracted using the flowCore package for R.[82] Plots were produced using Prism 6, Illustrator CS6 v16.0.3 (Adobe), Photoshop CS6 (Adobe), and R using the gplots, ggplot2 and circlize package. [83–85]

4. Results

4.1 B cell memory against *Kp* O-antigen

In order to isolate B cells expressing *Kp* O-antigen-binding antibodies biotinylated O-antigen baits were used in flow cytometry. To exclude the possibility of unspecific binding, lipid A and the vast majority of common part was completely replaced by a single biotin molecule. The analysis was focused on B cell memory against the two structurally distinct and clinically most relevant *Kp* O1 and O3 O-serotypes (Fig. 5A). Using fluorescence-labeled streptavidin conjugates, O1 and O3 O-antigen-binding memory B cells were detected in peripheral blood of the vast majority of individuals (Fig5B and 5C). Prior occupation of the BCR by binding to non-biotinylated O-antigen reduced the frequency of O3 O-antigen-binding memory B cells to baseline (Fig.5D). The data show that the biotinylated O1 and O3 O-antigen baits are suitable to identify and isolate O-antigen-binding B cells and suggest BCR-mediated binding of O-antigen.

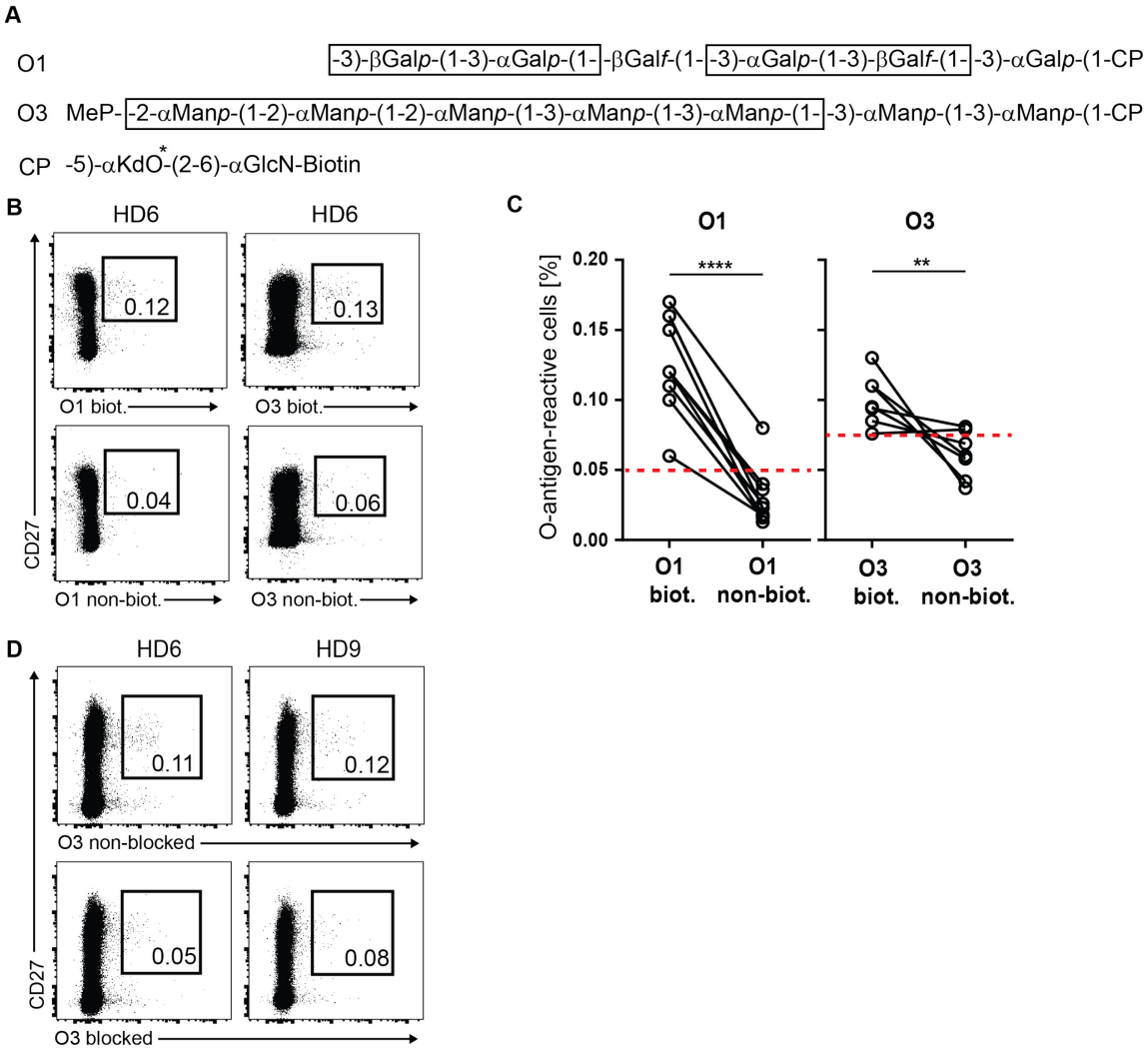


Figure 5 | Biotinylated O-antigen identifies O1 and O3 O-antigen-binding memory B cells by flow cytometry (A) Schematic chemical structure of biotinylated Kp O1 and O3 O-antigen baits (J. Lukasiewicz, unpublished). Repetitive units are boxed; CP: common part; * = (4-1) L-glycero-D-manno-heptose or hydrogen. (B) Representative flow cytometry of single, 7-AAD$^-$, CD19$^+$ cells from peripheral blood binding to biotinylated (top) or non-biotinylated (bottom) O1 (left) and O3 (right) O-antigen fractions from donor HD06. (C) Frequency of O-antigen-binding cells gated as in B from eight individuals for O1 (left) and O3 (right) O-antigen. Red line indicates positive cut-off. (D) Frequency of O3 O-antigen-binding B cells previously incubated with non-biotinylated O3 O-antigen (bottom) or with PBS (top) for donor HD06 and HD09. Data is representative of at least two experiments. Paired Student's *t* test was used for statistical analysis.

4.2 Ig gene characteristics of *Kp* O-antigen-binding memory B cells

To characterize the molecular features of the Ig genes from O1 and O3 O-antigen-binding memory B cells single B cell Ig gene sequencing was used.[76–78] Thus, *IGH* and the corresponding *IGK* or *IGL* transcripts from single cell sorted live, single, $CD19^+$, $CD27^+$, O1 and O3 O-antigen-binding memory B cells from the peripheral blood of 8 human volunteers were amplified and sequenced (Supplementary Fig. 1). Ig gene sequences revealed that the O-antigen-binding B cell repertoire was dominated by non-class switched B cells expressing *IGHM* transcripts (Fig.6A and Supplementary Fig. 2A). Antigen-specific B cell repertoires can be biased towards usage of a certain Ig gene or Ig gene family.[86,87] Therefore, Ig genes of O-antigen-binding B cells were identified by aligning them to the IMGT Ig receptor germline database.[88] Irrespective of the Ig isotype, O-antigen-binding B cells showed a predominant usage of the large IGHV3 Ig gene family (Fig6B and Supplementary Fig. 2B).

B cells showing the same IGHV gene, IGHJ gene, HCDR3 length and >90% HCDR3 similarity, and sharing identical somatic hypermutations in the IGHV gene (here referred to as B cell cluster) mark common ancestry and suggest selection. O1 and O3 O-antigen-binding B cell cluster were identified in the vast majority of individuals (Fig. 6C and Supplementary Fig. 2C) The vast majority of IGHV genes showed somatically mutated Ig genes in a range comparable to published total IgM, IgA, and IgG memory B cell repertoires (Fig. 6D and Supplementary Fig. 2D).[39,89,90] Together, the Ig gene characteristics of O1 and O3 O-antigen-binding B cells confirm their memory status and suggest that O1 and O3 O-antigen memory B cells have been selected within the GC reaction, and therefore, express affinity-matured antibodies.

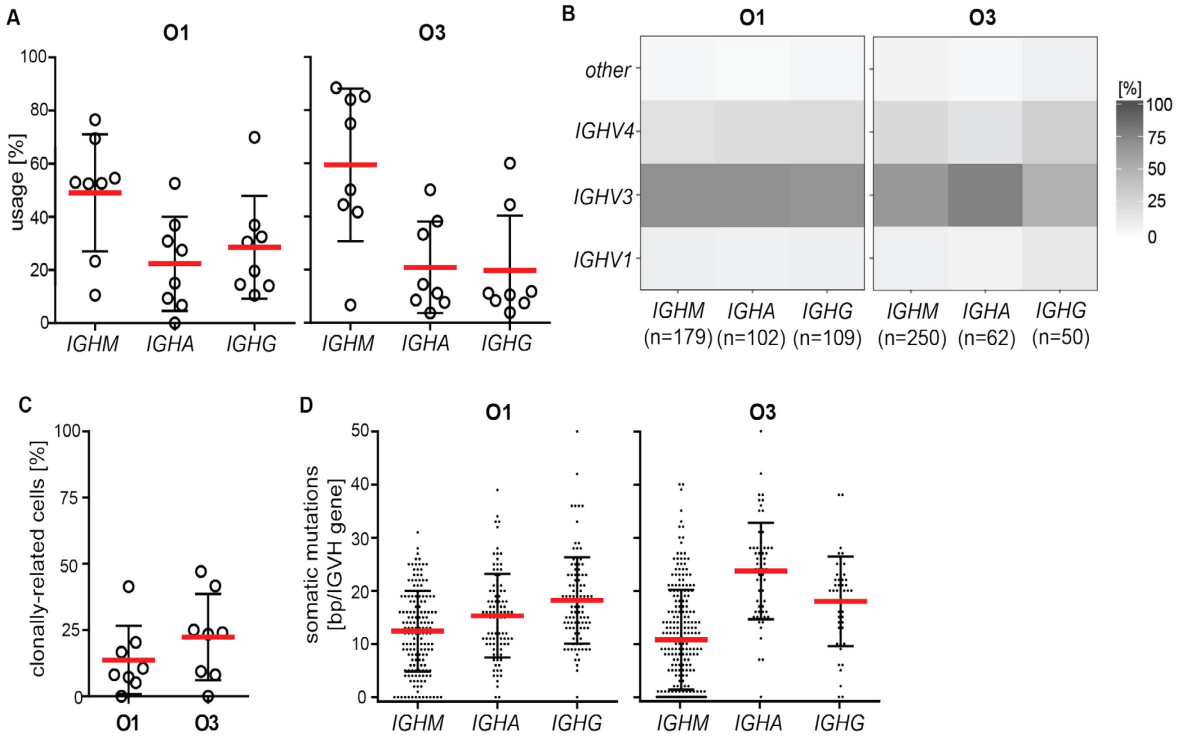


Figure 6 | Ig gene characteristics of *Kp* O1 and O3 O-antigen-binding antibodies. Ig gene analysis from single cell sorted O1 (left) and O3 (right) O-antigen-binding memory B cells. Percentage of (A) the Ig isotype determined by Ig gene sequencing, (B) the *IGHV* gene family per Ig isotype and (C) the overall degree of clonal expansion per individual. (D) Number of somatic hypermutations per *IGHV* gene. Red bars indicate mean; error bars show standard deviation.

4.3 O1 and O3 O-antigen-binding memory B cells express affinity-matured antibodies

To determine if O-antigen-binding memory B cells express affinity-matured antibodies, 40 and 41 antibodies from O1 and O3 O-antigen memory B cells respectively, were randomly selected, cloned, and recombinantly expressed. Donors that predominantly expressed O1 and O3 O-antigen IgM B cell memory were preferentially chosen, as the O1 and O3 O-antigen memory B cell repertoire is dominated by IgM-expressing memory B cells (Fig7A). For better comparison, all antibodies were expressed as IgG1, irrespective of the original isotype of the O-antigen-binding cell. ELISA could identify a fraction of antibodies binding the biotinylated O1 and O3 O-antigen bait (Fig.7B). The binding was confirmed to whole O1 and O3 LPS by Immunoblot (Fig7C). To confirm that

these antibodies specifically bind the O-antigen baits and not Streptavidin used for FACS sorting and ELISA immobilization binding to non-labeled Streptavidin was tested by ELISA. O1 antibodies did not show binding to Streptavidin and only a minor fraction of O3 O-antigen-binding antibodies showed binding to Streptavidin in ELISA (Fig7D). Hence, the data suggest that our sorting strategy, while enriching for B cells with O-antigen-specific antibodies, also isolated B cells whose antibodies bind either to Streptavidin or are poly-reactive, a prior observed feature in human peripheral memory B cells.[39,89,90] To assess this, binding of O3 antibodies to the unrelated human protein Insulin, was tested by ELISA. Most antibodies that bound Streptavidin, bound as well to Insulin confirming the non-specificity of these antibodies (Fig7E and Supplementary Table 2). Noteworthy, monoclonal antibody generation and reactivity measurements were necessary to determine the specificity of the antibodies as cells bearing specific BCRs could not be separated from non-specific ones by the fluorescence intensity obtained for O-antigen in flow cytometry (Fig7F). In total 6 and 10 specific antibodies to O1 and O3 O-antigen were identified, respectively. This included one and two antibodies for O1 and O3 O-antigen, respectively, which bound in Immunoblot to whole LPS but not by ELISA. Surprisingly, specific antibodies only originated from IgM or IgA-expressing memory B cells which was confirmed (except for KePBO1-093 which was IgA/IgG double-positive) by their surface Ig isotype expression level using the fluorescence signals from indexed flow cytometry data (Fig7G and Fig7H). Together, the data show that O1 and O3 memory B cells expressing specific antibodies (here referred to as O-antigen-specific memory) can be found within the IgM and IgA memory B cell compartment.

During affinity maturation B cells undergo clonal expansion and acquire SHM. Hence, we determined if O-antigen-specific memory B cells have a higher likelihood to be part of a B cell cluster and show somatically mutated Ig genes. Indeed, O1 and O3 O-antigen specific memory was enriched in B cell clusters (Fig7I). In addition to that they showed normal to elevated level of SHM, especially in IgM memory B cells, when compared to published SHM counts of total peripheral IgM populations (Fig7J).[39] To prove that the acquired mutations contribute to binding strength, SHM of one O1 and three O3 O-antigen-specific antibodies were reverted to their predicted germline configuration. Compared to their mutated counterpart, all germline antibodies showed loss of binding

to O-antigen, in ELISA (Fig7K). The data shows that peripheral O-antigen-specific memory B cells underwent clonal expansion and acquire mutations that are crucial for O-antigen-binding, thus, express affinity-matured antibodies.

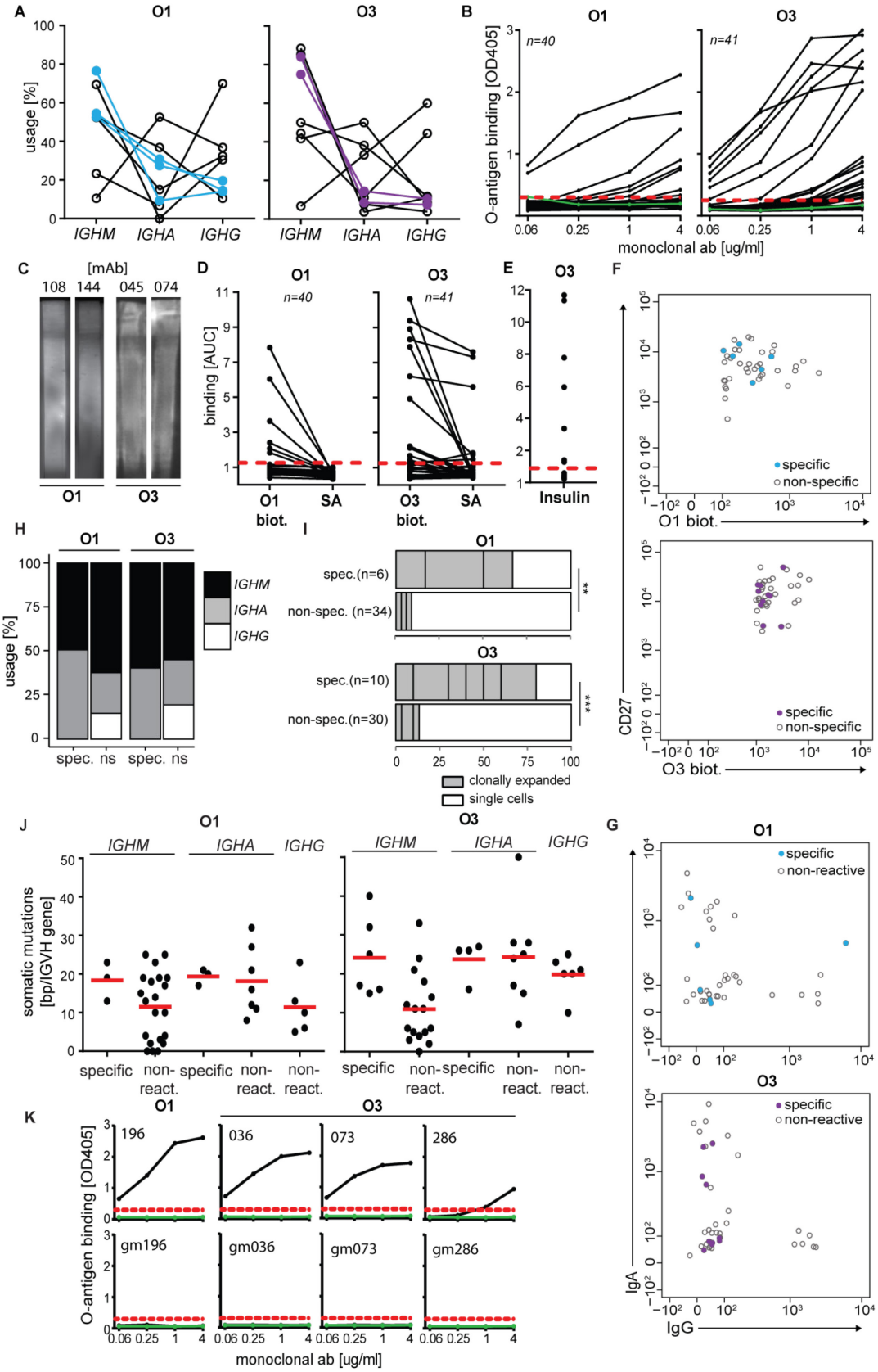

A
O1
O3
usage [%]
IGHM
IGHA
IGHG
B
O1
O3
n=40
n=41
O-antigen binding [OD405]
monoclonal ab [ug/ml]
C
[mAb]
108 144 045 074
O1
O3
D
O1
O3
n=40
n=41
binding [AUC]
O1 biot.
SA
O3 biot.
E
O3
Insulin
F
specific
non-specific
O1 biot.
CD27
O3 biot.
H
O1
O3
usage [%]
IGHM
IGHA
IGHG
spec.
ns
I
O1
spec.(n=6)
non-spec. (n=34)
O3
spec.(n=10)
non-spec.(n=30)
**
***
clonally expanded
single cells
J
O1
O3
IGHM
IGHA
IGHG
somatic mutations [bp/IGVH gene]
specific
non-react.
G
O1
O3
specific
non-reactive
IgA
IgG
K
O1
O3
196
036
073
286
gm196
gm036
gm073
gm286
O-antigen binding [OD405]
monoclonal ab [ug/ml]

Figure 7 | O-antigen-specific memory B cells express affinity-matured antibodies. (A) Monoclonal antibodies from O1 (left) and O3 (right) O-antigen-binding memory B cells were generated from three (light blue) or two (purple) donors showing a BCR repertoire dominated by IgM memory B cells and tested for (B) binding to the biotinylated O-antigen bait in ELISA, (C) whole LPS by Immunoblot (D) Streptavidin in ELISA and (E) recombinant human Insulin by ELISA. (F) Fluorescence intensity index data recorded for Streptavidin-A647 (O-antigen; x-axis) from single cell sorted O1 (top) and O3 (bottom) O-antigen-binding memory B cells with O-antigen-specific (O1, light blue circles; O3, purple circles) and non-specific (empty circles) memory B cells. (G) Isotype usage as determined by fluorescence intensity index data for O1 (top, light blue) and O3 (bottom, purple) O-antigen-specific antibodies. (H) Isotype expression, (I) frequency of clonally-expanded cells and (J) level of somatic hypermutation of O-antigen binding memory that express specific or non-specific antibodies as determined from (A-E). (K) ELISA of mutated antibodies (top) and their corresponding germline form (bottom). Statistics were performed using Fisher-Exact; Green line: mGO53 negative control antibody; red dashed line: negative cut-off; Data is representative of at least two experiments.

4.4 Peripheral O3 O-antigen B cell memory is clonally-related to intestinal effector B cells

Peripheral O-antigen-specific B cell memory showed partial isotype-switching to IgA suggesting a mucosal origin of these cells. Further, *Kp* frequently colonizes the intestinal tract where specific IgA responses against bacterial antigens are induced.[91] LP plasmablasts still express surface BCR, thus, to determine if a clonal relationship between peripheral memory B cells and lamina propria plasmablasts can be observed, live, single, 7-AAD-, CD19+, O3 O-antigen-binding cells from phenotypically healthy lamina propria of three donors (HD02, HD05 and HD10) were isolated by FACS (Fig8A). To evaluate if O-antigen-binding LP plasmablasts possess similar Ig gene characteristics than peripheral O-antigen-binding memory, IgH and IgL genes from single cell sorted O3 O-antigen binding LP plasmablasts were amplified and sequenced. LP plasmablast predominantly expressed *IGHA* transcripts and the majority of cells used *IGHV3* family gene segments (Fig.8B and Fig.8C). All antibodies showed somatically mutated *IGHV* regions within the range of randomly assessed published LP plasmablast repertoires (Fig.8D).[51] Further, overlapping B cell clusters between peripheral IgM, IgA, and IgG memory and the IgA-expressing LP plasmablasts were identified (Fig8E). Together, the data show that the systemic and intestinal response against O3 O-antigen is clonally-

related and suggest that, similar to peripheral O-antigen-specific memory, O3 O-antigen-specific LP plasmablasts express affinity-matured antibodies.

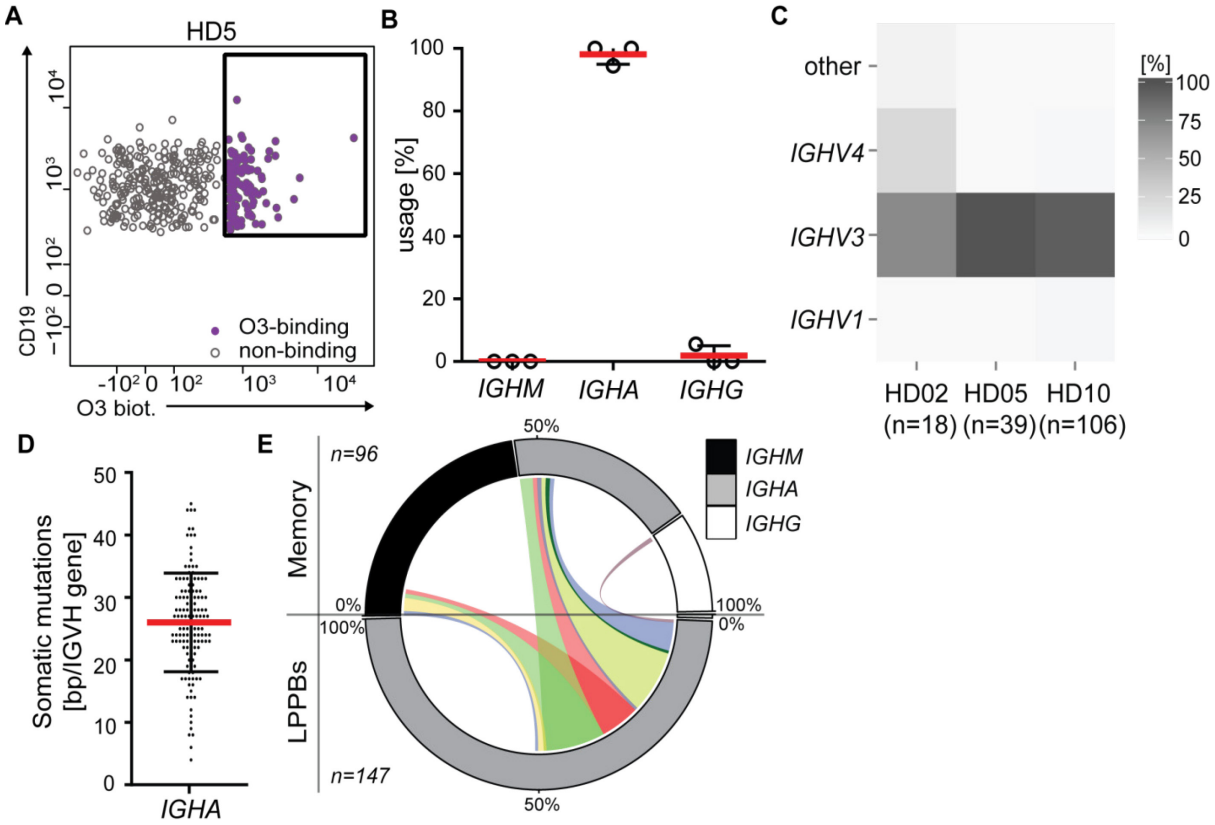


Figure 8 | O3 O-antigen-binding peripheral memory and LP plasmablasts are clonally-related. (A) Representative sorting strategy of single, 7-AAD-, CD19+, O3 O-antigen-binding (purple) LP plasmablasts of healthy donor 05 (HD05); sorting gate is shown in bold black; negative population is shown for comparison (grey open circle). (B) Ig isotype of O3 O-antigen binding B cells as determined by sequence (C) Number of IGHV somatic hypermutations (D) Each B cell cluster showing at least one member in both compartments is connected by a colored line, data are pooled from healthy donors 02, 05 and 10. Red bars indicate mean; error bars show standard deviation.

4.5 Intestinal O-antigen-specific antibodies are affinity-matured

To test whether LP plasmablasts express affinity matured O3 O-antigen-specific antibodies 41 antibodies from one donor were expressed and assayed for binding to O3 O-antigen, by ELISA (Fig.9A). 32 out of 41 antibodies showed binding to the biotinylated

O3 O-antigen bait and the vast majority was identified to be specific as assessed by binding to Streptavidin (Fig.9B). As shown previously for peripheral *Kp* O-antigen-specific antibodies, the reverted germline counterparts of two specific antibodies from LP plasmablasts completely lacked binding to O3 O-antigen in ELISA (Fig.9C). The data shows, similar to their peripheral memory counterpart, that O-antigen-specific LP plasmablasts can express antibodies that acquire mutations that are crucial for O-antigen-binding, and therefore, are affinity-matured.

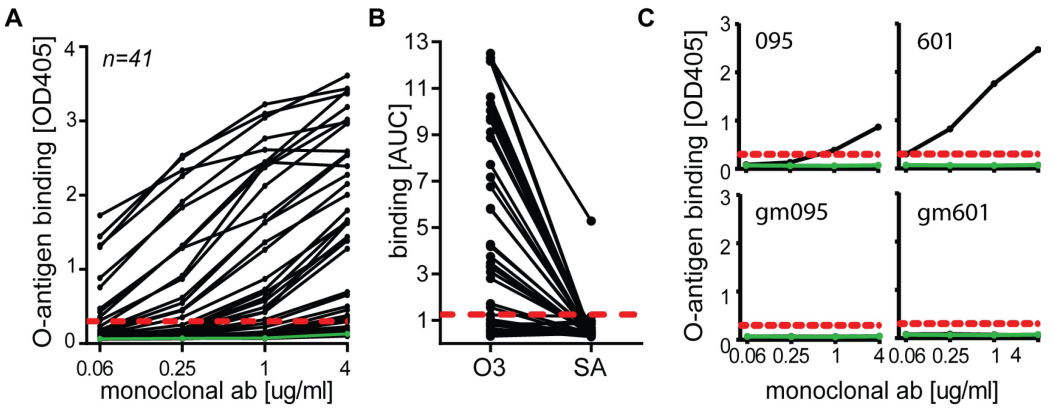


Figure 9 | O3 O-antigen-binding LP plasmablasts express affinity-matured antibodies Binding of monoclonal antibodies isolated from O3 O-antigen-binding LP plasmablasts to (A) biotinylated O-antigen and (B) Streptavidin in ELISA. (C) Binding of antibody 095 and 601 in their mutated (top) or germline (bottom) form to biotinylated O3 O-antigen in ELISA. Red dashed lines show negative cut-off, green line shows mGO53 as negative control; AUC = Area under curve; OD = optical density; gm = germline.[78] Data is representative of two experiments.

4.6 O3 O-antigen specific antibodies cross-bind to mannan-based *Kp* O-serotypes

Other *Kp* O-serotypes express mannan-based O-antigen structures that are highly similar to O3 O-antigen. Compared to O3 O-antigen, mannan-based O-serotype variants are composed of a tetrameric repeat (O3a) and a trimeric repeat (O3b) lacking either one or two α-mannoses connected by 1-2 linkages, respectively, or a trimeric repeat containing a different anomer and an 1-2 instead of an 1-3 linkage (O5) (Fig.10A). To

test if the collection of O3 O-antigen-specific antibodies bound to any of the variants, antibody binding to whole *Kp* O3a, O3b, and O5 LPS was determined by Immunoblot (Fig.10B and Supplementary Table 2 and 3). The data showed that all O3 O-antigen-specific antibodies bound to whole O3a LPS. Further, antibodies additionally binding to O3b or O5 or both O-serotypes were identified. Surprisingly, within the O3 collection, antibodies that cross-bound the same variants could even show different binding profiles (compare antibody 666, 694 and 191 in Fig.10B). The data demonstrates that O3 O-antigen-specific antibodies bind to and, just as crucial, can discriminate between highly similar mannan-based *Kp* O-antigens, strongly suggesting that each antibody targets its epitope with high specificity (here termed cross-specific binding).

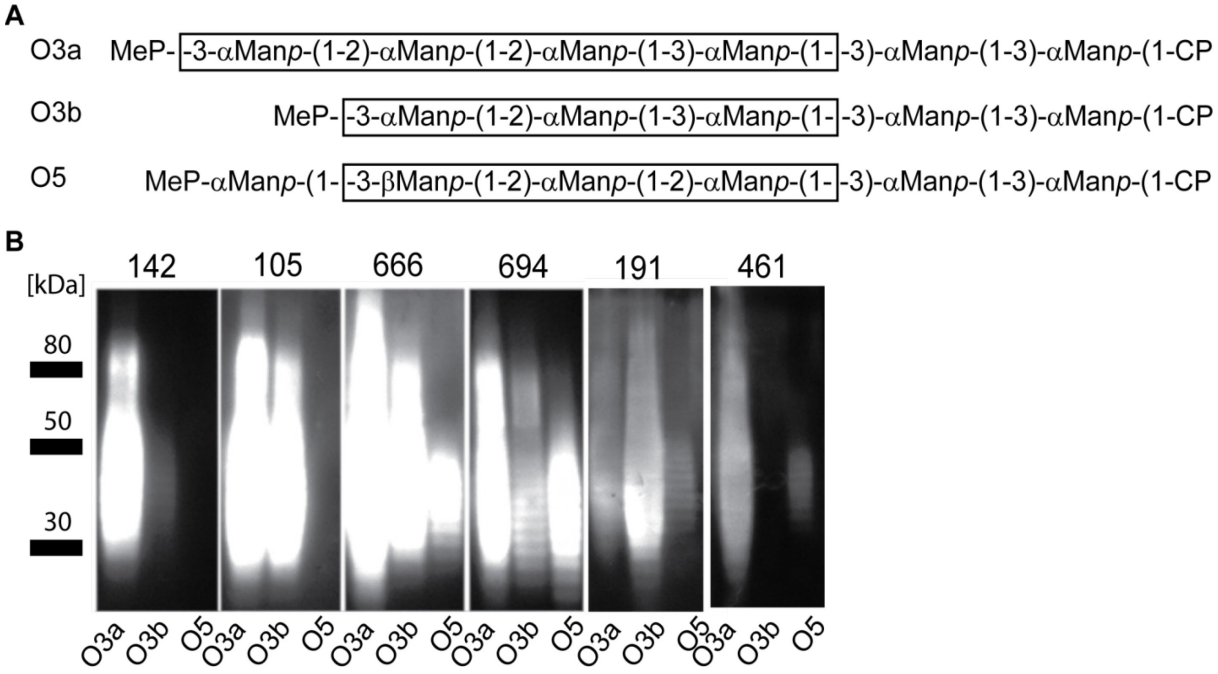
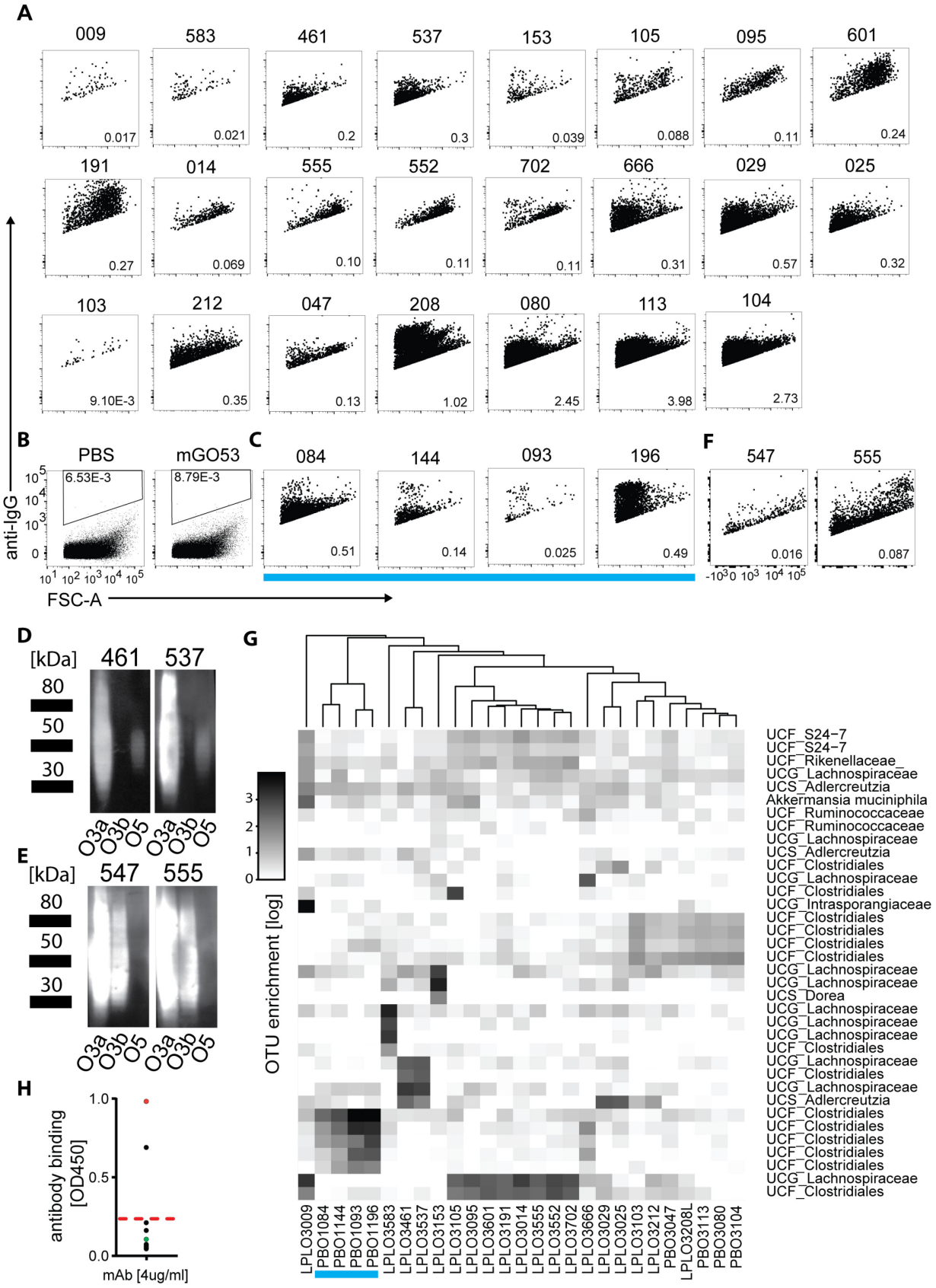


Figure 10 | O3 O-antigen-specific antibodies cross-bind *Kp* mannan-based LPS variants (A) Linear chemical structure of mannan-based *Kp* LPS O-serotypes with a tetrameric repeat (O3a), trimeric repeat (O3b) and trimeric repeat containing an anomer and a αMan*p*1-2αMan*p* instead of a αMan*p*1-3αMan*p* linkage (O5). (J. Lukasiewicz, unpublished) (B) Representative Immunoblots showing binding of O3 O-antigen-specific antibodies to *Kp* mannan-based whole LPS fractions of O-serotype O3a, O3b and O5. Numbers of representative antibodies are shown on top of each blot. Data is representative of at least two experiments.

4.7 O-antigen-specific antibodies bind taxonomically distinct microbes

To test whether the epitope targeted by the cross-specific antibodies is expressed and accessible on other microbes, a complex microbiome mixture (here termed microbiota) was isolated by intestinal lavage of four co-housed pair of mice. By flow cytometry, binding of O3 O-antigen cross-specific antibodies to the murine microbiota was assessed. For comparison O1 O-antigen-specific antibodies were included as controls. The antibodies showed different microbiota binding patterns and binding intensities (Fig.11A). The majority of antibodies bound less than 0.6%, however, antibodies binding up to 4% of the total microbiota were observed as well. Binding was Fc-independent as above background binding of a mature naïve control antibody expressed with a human IgG1 constant region was not detected (Fig.11B). As expected from their different glycan target, O1 O-antigen-specific antibodies showed a different microbiota binding pattern than O3 O-antigen-specific antibodies (Fig.11C). Further, the O3-cross-specific antibodies showed different binding patterns among themselves, suggesting binding to different bacteria. However, different antibodies also showed similar binding patterns. In line with this, O3 O-antigen specific antibodies showing highly similar microbiota binding pattern also had the same cross-binding pattern to mannan-based LPS fractions by Immunoblot (Fig.11A and Fig.11D). On the other hand, the same binding profile in Immunoblot could not predict the binding profile to the microbiota. For example, two antibodies 547 and 555 originate from the same B cell ancestor and show the same amino acid sequence except of one exchange in their kappa light chain at position 54. This mutation resulted in the exchange of the germline-encoded Tyrosine to Proline or Serine, Y54P or Y54S, respectively. Both antibodies, showed binding to O3a and O3b by Immunoblot in a similar fashion (Fig.11E). However, the Y54P mutation reduced binding to the microbiota, illustrating that a single mutation can have strong implications on antibody binding properties (Fig.11F). To determine if antibodies showing different microbiota binding patterns in FACS bind to different members of the microbiota, antibody-bound bacteria were sorted by FACS and the V3-V4 region of their 16S ribosomal DNA (rDNA) was amplified and sequenced by PCR and Illumina sequencing, respectively. Using the V3-V4 16S rDNA sequences, the taxonomy of the antibody-bound bacteria was identified by sequence homology to the 16S rDNA

Greengenes database.[92] At least one antibody of each microbiota binding pattern was included in the experiment. The obtained 16S rDNA sequences were assembled into operational taxonomic units (OTU) of >99% sequence similarity to the 16S rDNA reference database and each antibody-bound population was screened for enriched OTUs (> 4-fold enrichment) (Fig.11G). O1 O-antigen-specific antibodies enrich for different OTUs than O3 cross-specific antibodies. Furthermore, antibodies showing similar FACS microbiota binding profiles also enriched for the same OTUs, validating the methodology. On the other hand, antibodies showing different microbiota binding pattern enriched for different OTUs that could even belong to different phyla, suggesting that the targeted epitope is present and accessible on other microbes. However, the most identified OTUs were unclassified families of the *Clostridiales* order including unclassified genera of the *Lachnospiricaea* family, which are also found to be highly abundant in the human intestinal tract.[93] *Kp* was not identified within the whole microbiota. This led to the conclusion that anti-O-antigen antibodies can cross-specifically bind to non-*Kp* microbes. However, mannan glycan structures are not only found on bacteria but also in the cell wall of commensal fungi, e.g. *Sacharomyces cerevisiae* (*Sc*).[94] Indeed, by ELISA, one antibody was found to bind to *Sc* cell wall as well (Fig.11G and Supplementary Table 2 and 3). Altogether, the data show that naturally acquired O1 and O3 O-antigen antibodies can cross-specifically bind to taxonomically distinct members of the microbiome.


A
009
583
461
537
153
105
095
601
0.017
0.021
0.2
0.3
0.039
0.088
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014
555
552
702
666
029
025
0.27
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0.10
0.11
0.11
0.31
0.57
0.32
103
212
047
208
080
113
104
9.10E-3
0.35
0.13
1.02
2.45
3.98
2.73
B
PBS
mGO53
6.53E-3
8.79E-3
anti-IgG
FSC-A
C
084
144
093
196
0.51
0.14
0.025
0.49
F
547
555
0.016
0.087
D
[kDa]
461
537
80
50
30
O3a
O3b
O5
E
[kDa]
547
555
80
50
30
O3a
O3b
O5
G
OTU enrichment [log]
3
2
1
0
LPLO3009
PBO1084
PBO1144
PBO1093
PBO1196
LPLO3583
LPLO3461
LPLO3537
LPLO3153
LPLO3105
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LPLO3029
LPLO3025
LPLO3103
LPLO3212
PBO3047
LPLO3208L
PBO3113
PBO3080
PBO3104
UCF_S24−7
UCF_S24−7
UCF_Rikenellaceae_
UCG_Lachnospiraceae
UCS_Adlercreutzia
Akkermansia muciniphila
UCF_Ruminococcaceae
UCF_Ruminococcaceae
UCG_Lachnospiraceae
UCS_Adlercreutzia
UCF_Clostridiales
UCG_Lachnospiraceae
UCF_Clostridiales
UCG_Intrasporangiaceae
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UCF_Clostridiales
UCF_Clostridiales
UCF_Clostridiales
UCF_Clostridiales
UCF_Clostridiales
UCG_Lachnospiraceae
UCF_Clostridiales
H
antibody binding [OD450]
1.0
0.5
0.0
mAb [4ug/ml]

Figure 11 | O-antigen-specific antibodies bind taxonomically distinct microbes Flow cytometry dot plots showing (A) the antibody-bound population (microbiota binding pattern) of O3 O-antigen-specific antibodies within 2 x 10^6 recorded events pregated on forward scatter > 60, (B) the frequency of antibody bound bacteria using an isotype control antibody (mGO53) expressed as human IgG1 or PBS (C) the antibody-bound population of O1 O-antigen-specific antibodies within 2 x 10^6 recorded events pregated on forward scatter > 60. Immunoblot showing the binding of antibodies (D) 461 and 537 and (E) 547 and 555 to whole LPS of the mannan-based O-serotypes O3a, O3b and O5. (F) Flow cytometry dot plots depicting the antibody-bound population (microbiota binding pattern) of the O3 O-antigen-specific cluster antibodies 547 and 555; frequency is shown within the graph; antibody names are depicted on the top of each graph. (G) Enrichment of operational taxonomic units (OTUs) of O1 and O3 O-antigen-binding antibodies compared to the whole microbiota hierarchically clustered according to similarity; samples were FACS sorted as shown in (B); UC: unclassified; F: family; G: genus; S: species; depleted antibody OTU combinations are shown as 0; antibody names are given below; LPL and PB depicts antibodies originating from lamina propria and peripheral blood, respectively; light blue line shows O1 O-antigen-specific antibodies (H) Binding of O3 O-antigen-specific antibodies to *Sc* cell wall by ELISA, green dot and red dot show negative control and positive control serum, respectively; red dashed line shows negative cut-off. Data is representative of two (A-F, H) or one experiment (G).

5. Discussion

5.1 O-antigen specific antibodies can be isolated using biotinylated O-antigen and identified using single cell Ig gene sequencing

Antibodies against glycan antigens are potentially promising candidates for current or future passive immunization strategies against bacteria or cancer.[95] There are two major challenges in developing appropriate therapeutic antibodies against glycan antigens: identification of relevant glycan targets and isolation of potential therapeutically active antibodies. Using *Kp* LPS O-antigens, specific B cell memory can be enriched by single B cell sorting in combination with biotinylated O-antigen. Downstream antibody cloning, expression, and reactivity measurements identified antibodies specifically targeting the O-antigen baits. A few Ig gene sequence characteristics could already reveal and enrich for specific antibodies. They originated from IgM- or IgA-expressing memory B cells with somatically mutated BCRs, showing prior clonal expansion. Here, these criteria were found for *Kp* O1 and O3 O-antigen, two structurally distinct glycans, suggesting that these criteria might apply to antibodies against other glycan antigens of commensal bacteria and could be used by others to enrich for affinity-matured anti-glycan antibodies. Noteworthy, these criteria apply to peripheral blood of healthy humans without any former infection or immunization history, a cheap and easy-to-obtain source of memory B cells.

5.2 Peripheral *Kp* O-antigen-specific B cell memory express IgM or IgA antibodies

O-antigen specific memory was only identified in IgM and IgA peripheral memory B cells, but not in IgG memory B cells, although IgG memory has been predominantly used to identify affinity-matured antibodies.[43,96,97] The data suggest that under natural non-inflammatory conditions O-antigen-specific memory does not undergo CSR or switches to IgA. The former was expected, as it has been demonstrated that peripheral IgM memory is involved in the humoral immune response to the encapsulated bacteria *Streptococcus pneumoniae.*[57] The latter might be explained by their clonal relationship

to mucosal antibody responses. Peripheral IgM- and IgA-expressing cells were found within the same B cell cluster. However, comparisons of total peripheral IgA and IgM memory B cell repertoires revealed that clonal relations between both populations are rare.[98] Thus, it remains open if this clonal relationship is specifically observed in the naturally acquired antibody responses to glycan antigens of commensal bacteria. Further, in contrast to the majority of donors whose O-antigen-binding B cell repertoires were dominated by IgM memory, single individuals predominantly possessed IgA or IgG-expressing O-antigen-binding memory B cells. Thus, the colonization route, infection history and current immune status might define the Ig subclass distribution of the O-antigen-binding B cell repertoires. The data suggest that future studies on anti-bacterial glycan antibodies should not disregard peripheral IgM and IgA B cell memory.

5.3 The vast majority of individuals naturally acquire peripheral *Kp* O-antigen-binding B cell memory

The data showed that the vast majority of donors possess peripheral O-antigen-binding memory B cells which harbor affinity-matured *Kp* O-antigen-reactive antibodies. On average and subtracting background, one in two-thousand peripheral B cells showed binding to O1 and O3, a frequency similar to tetanus toxoid-binding memory B cells in a vaccinated population under steady-state conditions.[99] This let us suggest that almost every individual is currently or has been exposed to O1 and O3 *Kp* O-serotypes or that exposure to the same or similar glycan epitopes on other bacteria or fungi resulting in *Kp* O-antigen cross-binding B cell memory. The data suggest that both explanations are not mutually exclusive. However, antibody reactivity measurement revealed that only one in approximately 10.000 memory B cells harbor specific antibodies to O1 or O3 O-antigen, as measured by ELISA and Immunoblot. Further, the antibodies from different donors showed different binding strength by ELISA, suggesting that the quality of the response still differs between donors. Despite this, a substantial fraction of antibodies from O-antigen-binding memory B cells did not bind to O-antigen in ELISA and of those, only two antibodies showed weak binding to O-antigen in a more sensitive method,

such as Immunoblot. However, they could not be distinguished from cells bearing specific BCRs, by their fluorescence intensity obtained for O-antigen in flow cytometry. This phenomenon could not be solely explained by poly-reactive binding properties of the antibodies or background staining in flow cytometry. Interestingly, among the non-reactive antibodies isolated using the O1 O-antigen bait (which does not contain the 1-4 branching Galactose as O1+ or O2+), two antibodies were identified that specifically bound biotinylated galactan-based O2+ but not O1 or O2 O-antigen by ELISA (Supplementary Table 1 and data not shown). Similarly, using the O3 O-antigen bait, an antibody showing no reactivity to O3 O-antigen by ELISA or Immunoblot but reacting to the mannan-based O5 O-antigen by Immunoblot was identified (Supplementary Table 3 and data not shown). A likely explanation for this observation would be that repetitive antigens (such as O-antigens) could theoretically engage multiple BCRs on a single cell that show low affinity binding, resulting in sufficient avidity to bind the antigen *ex vivo*. Thus, a B cell harboring a BCR that targets a given repetitive structure with high affinity, e.g. O5 O-antigen, could potentially still be activated using a similar glycan structure, e.g. O3 O-antigen that is bound with low affinity.

5.4 T cell-dependency of B cell memory formation against *Kp* O-antigens

Plain polysaccharide vaccines poorly elicit memory formation in humans which can wane rapidly, resulting in loss of protection.[100] Polysaccharide vaccines conjugated to a protein carrier (e.g. diptheria toxin) can overcome this limitation by efficiently generating T-cell dependent antibody responses, resulting in long-term memory.[100] Further examples of T cell-dependent antibody responses against bacterial glycans have been reported, such as repetitive glycans with zwitterionic properties can be presented onto MHC class II molecules to T cells and in turn enable T cell help.[101] *Kp* O-antigen does not possess similar zwitterionic properties and is thus supposed to be a T cell-independent antigen. Direct evidence to support this, however, is lacking. O-antigen-specific B cells showed the same characteristics (mutated Ig genes, possibly class-switched Ig, and clonally expanded) as B cell memory against classical T cell-dependent antigens. Further, reverting O-antigen-specific antibodies to their predicted germline

configuration showed that the mutations are crucial for their higher binding strength. This strongly suggests that naturally acquired human anti-glycan B cell memory does develop depending on T cell-dependent selection forces. A suggestion already made by Fiskesund *et al* by identification of somatically-mutated naturally-occurring antibodies binding to the TI human autoantigen phosphorylcholine (PC).[52] In line with this, B cells, with help of LPS-specific BCRs, are capable to ingest whole bacteria and present bacterial peptides onto their MHC class II complexes.[102] Therefore, even if their BCR is directed against LPS, these cells could potentially receive T cell help and affinity-mature within GCs (Fig.12). However, indirect antigen presentation would be less efficient than direct presentation (e.g. as observed in immunizations with single protein antigens) as multiple cognate TFH T cell receptor specificities and not only one (given that the target protein would possess only one T cell epitope) is needed to provide efficient T cell help. Further studies need to determine if the generation of anti-glycan antibodies under natural circumstances is T-cell dependent.

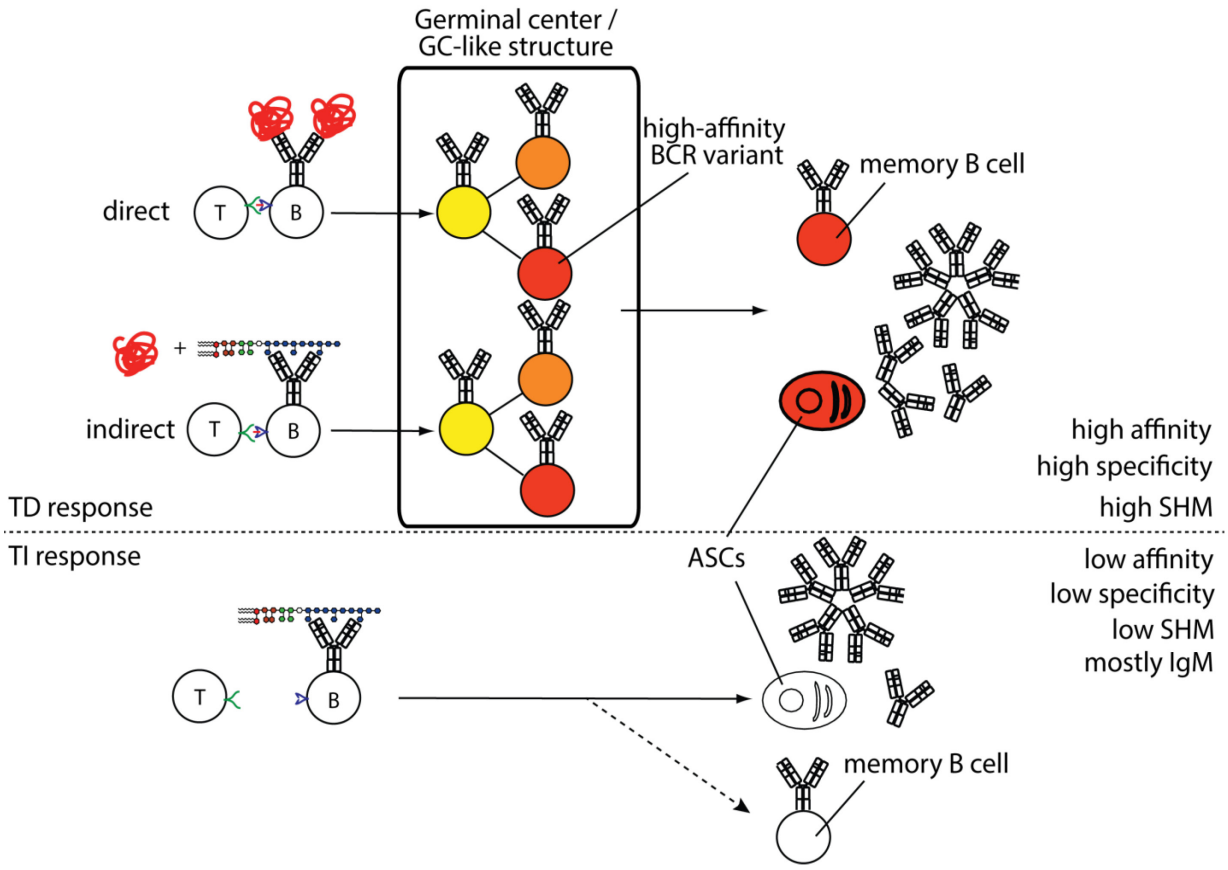

Figure 12: B cells harboring anti-glycan antibodies could potentially undergo T-cell dependent affinity maturation by indirect presentation of bacterial peptides (middle). Despite TD (top) and TI (bottom) responses, B cells with anti-glycan antibodies could internalize not only the glycan moiety, but additional proteins, which could be presented to T cells and elicit T cell help. Subsequently, this would enable them to undergo affinity maturation in GCs.

5.5 O-antigen-specific B cell memory formation might require long-term exposure

Reports suggest that anti-glycan antibody responses are inefficiently formed and might take long-time and constant exposure to be established. [103,104] Taking into account that O-antigen specific peripheral memory showed average to high levels of SHM, these antibodies could have been maintained long-term in the system. Another possibility how this high level of SHM could arise comes from a study in humans who are unable to mount GC responses due to CD40L mutations. In these patients, Weller *et al* identified somatically mutated BCRs in marginal zone (MZ) B cells.[105] Their data suggested that GC-independent diversification of Ig genes in MZ B cells are responsible for bacterial recognition. The crucial role of mutations in the IGVH gene in their specificity in O-antigen-recognition makes it unlikely that O-antigen-specific memory cells derive from MZ B cells that randomly pre-diversified the Ig gene region. However, this does not exclude the possibility that O-antigen-specific B cells are part of the circulating MZ B cell compartment. Thus, it is unclear which effect impaired CD40/CD40L interaction has on antibody affinity maturation in these patients and future studies need to refine how efficiently anti-glycan antibodies are naturally generated.

5.6 Peripheral anti-glycan memory B cells might act as a reservoir for intestinal antibody responses

The clonal relationship of intestinal and peripheral IgA B cell repertoires has been previously established.[106] Here, the relationship between IgA intestinal effector and IgA peripheral memory B cells for O3 O-antigen was confirmed in humans. Despite this, the data also showed a clonal overlap between LP plasmablasts and peripheral IgM memory

B cells, a relationship rarely observed.[106,107] Hence, it remains elusive if this relationship predominantly applies to glycan antigens or in particular O3 O-antigen. It has been demonstrated that the intestinal B cell repertoire remains stable and is recalled following experimental B cell depletion.[108] Further, memory B cells can leave and re-enter GCs of PPs and are thus capable of synchronizing intestinal antibody response.[109] The data supports the notion that peripheral memory B cells can act as a reservoir for intestinal antibody responses. However, CSR is an irreversible process and O-antigen-specific IgM cells in the intestinal lamina propria were not identified. The data suggest that IgM memory B cells could act as a reservoir for intestinal antibody responses and, additionally, peripheral IgA could also act as a reservoir for intestinal antibody responses or *vice versa*. If this holds true, mucosal vaccination could be enhanced by former induction of peripheral memory cells and boosted locally at intestinal surfaces. However, it remains to be studied if and how O-antigen-specific peripheral memory B cells, especially IgM memory cells, can be re-recruited into the lamina propria or if they possess inherent gut-homing capacities.

5.7 Affinity matured anti-glycan antibodies are found in the human lamina propria

Due to the high mutational load of human intestinal plasmablasts, it has been suggested that TI-antibody responses have a minor contribution to intestinal antibody responses in humans.[71] All anti-O-antigen antibodies obtained from LP plasmablasts showed mutated Ig genes, thus, the data questions the contribution of TD and TI antibody responses to the entire intestinal antibody responses. Further, it emphasizes that, in general, antibody responses against TI antigens cannot be distinguished from antibodies against TD antigens by mutational load and that differences between both need to be addressed on the antibody reactivity level. Apart from that, it supports the view that “innate” antibodies, as defined by unmutated antibodies targeting conserved glycan structures, do play a minor role in intestinal anti-glycan antibody responses in human adults. In line with this, it has been reported that SHM can occur outside fully developed intestinal GCs and lead to affinity-matured *Salmonella typhimurium* LPS-

binding antibodies.[110] If this pathway is involved in the generation of specific anti-glycan antibodies in humans remains to be studied.

5.8 Affinity maturation generates cross-specific antibodies

The human microbiome is distinct between individuals and among body sites of the same individual.[111] The individual microbiome remains relatively stable but can undergo temporal composition changes.[111] Microbial diversity on the serotype level or glycan diversity of the microbiome has not been determined but *Kp* alone comprises more than 80 different glycan structures suggesting that microbial glycans exceed the diversity of the human microbiome. Stool samples have been shown to harbor one of the highest diversity among the body habitats.[111] Counter-intuitively, unbiased intestinal antibody repertoires are oligoclonal, thus possess relatively reduced diversity.[51,107,109] The data showed that naturally acquired *Kp* O-antigen-specific antibodies cross-bind to other non-*Kp* microbes found in a complex mixture of microbes. These findings suggest that cross-specific binding of different members of the microbiome by anti-glycan antibodies could be a mechanism how the human immune system adapts and reacts to the glycan diversity offered by the microbiome (Fig.13). However, the overall contribution of cross-specific glycan antibodies in intestinal antibody responses in humans remains elusive. In line with this, former reports found that a small fraction of randomly cloned LP plasmablasts antibodies bound to more than one species of a relatively small panel of microbes, suggesting that cross-specific binding substantially contribute to the intestinal humoral immune response to a complex microbiota.[51] However, the functional relevance of cross-specific vs classical vs innate (in terms of poly-reactive and somatically mutated) antibodies in the humoral immune response to the microbiota needs to be determined. Further, whether affinity maturation preferentially selects B cells expressing antibodies, that target cross-binding epitopes over mono-specific ones, remains an interesting possibility.

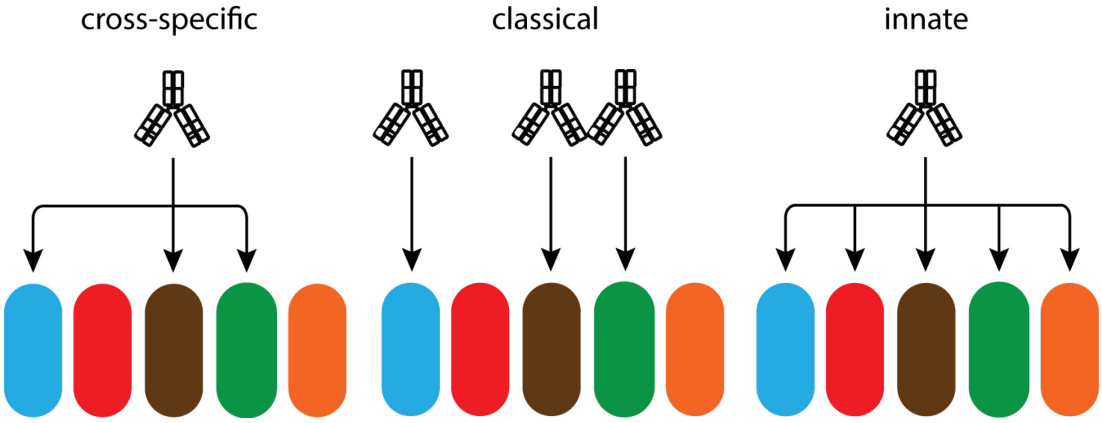

Figure 13: Extended binding model of antibodies to intestinal microbes. Despite, classical (middle) and innate (right) antibody binding of microbes the data suggest an additional model where anti-glycan antibodies cross-specifically bind taxonomically different microbes expressing the same or highly similar epitopes (left).

5.9 Naturally acquired anti-glycan antibodies are epitope-specific

As expected, anti-glycan antibodies can bind to the same epitope if present in two different structures.[112] However, binding can as well be heavily influenced by the context in which the epitope appears, suggestively by induced conformational changes that cannot be tolerated by the antibody antigen-binding site.[112] Using Immunoblot, O3 O-antigen-specific antibodies could discriminate between and specifically cross-bind to mannan-based *Kp* O-antigen variants, indicating that they target specific epitopes present in each structure. For example, antibody binding to O3 and O3a but not to O3b and O5 O-antigen or *Sc* cell wall suggests that antibodies showing this profile do bind an epitope within the αMan*p*1-2αMan*p*1-2αMan*p*1-3αMan*p* tetra-saccharide. This suggests that these antibodies could also bind to O-antigen of *Ec* O-serotype O9 and O9a, which are identical to *Kp* O3 and O3a respectively.[113,114] Furthermore, antibodies that bound O3, O3a, and O3b, but neither O5 O-antigen nor *Sc* cell wall, probably target an epitope within the αMan*p*1-2αMan*p*1-3αMan*p*1-3αMan*p* tetra-saccharide. Antibody binding profiles showed that one O-antigen structure can be preferentially bound with greater strength compared to others, e.g. 461 bound strongly to O3 and O3a, but only weakly to O5 O-antigen, and 694 strongly bound O3a and O5, but showed weaker staining of O3b O-antigen. This indicates that anti-glycan antibodies can tolerate small epitope

differences (anomer, linkage or only partially present epitopes). However, the respective drop in binding strength to similar structures argues for the high specificity of the antibodies to a given epitope. In line with this, only one antibody (208L) that bound all tested mannan-based structures was identified. The high specificity of the antibodies becomes even more apparent when comparing antibodies that showed the exact same binding profiles in Immunoblotting, but did not show the exact same microbiota binding pattern, e.g. antibody 105 and 601 bind O3, O3a and O3b, but not O5 O-antigen nor *Sc* cell wall, however, 105 enriches for an unclassified member of the *Clostridiales* family, which is completely absent in the antibody-bound fraction of 601. Altogether, this suggests that naturally acquired anti-glycan antibodies are highly specific and target epitopes that need to be resolved at molecular level.

5.10 Naturally-acquired anti-O-antigen antibodies could target a potential vaccine epitope

A major goal in vaccinology is the identification of appropriate antigen targets and, just as crucial, protective epitopes targeted by antibodies. Structure resolution, by methods such as co-crystallization, of antibody-antigen complexes can identify the epitope and crucial amino acid residues for interaction. However, O-antigens limit the use of direct measurements such as Hydrogen-deuterium-exchange mass spectrometry and co-crystallization because of their fast hydrogen exchange rates and heterogenous length distribution when extracted from bacteria cultures, respectively (German Cancer Research Center Core Facilities, personal communication). Further, solving the epitope using saturation-transfer-difference nuclear magnet resonance (STD-NMR) failed due to too high binding affinities of the antibody to O-antigen (J.Lukasiewicz, personal communication). The characterization of epitopes targeted by naturally acquired antibodies from healthy individuals could potentially reveal glycan epitopes which are preferentially targeted by the human immune system and epitopes to be avoided because of their potential to bind endogenous glycan structures.[115] The linear chemical structure of O3, O3a, and O3b O-antigen shows that within the tetrasaccharide αMan*p*1-2αMan*p*1-3αMan*p*1-3αMan*p* could lay a desirable target epitope because it could elicit cross-reactive antibodies to *Kp* O3, O3a, and O3b O-antigen. Binding to whole mannan-

based LPS fractions revealed several antibodies that could bind within this epitope. Thus, crystallization of these antibodies with a synthesized tetrasaccharide might reveal a potential vaccine epitope.

5.11 Passive immunization with anti O-antigen antibodies might have therapeutic potential to treat *Kp* infection

Antibodies against bacterial infections are a desirable tool to overcome multidrug-resistance but the identification of appropriate bacterial targets has been proven difficult due to high diversity. In contrast, few *Kp* O-antigen-specific antibodies could potentially cover the vast majority of clinical isolates, including the wide-spread endemic serotypes. The identified fully human candidate antibodies might be used to prevent or treat *Kp* infections of the O1 or O3, O3a, and O3b O-serotypes, comprising around half of the clinical isolates.[12] Indeed, full protection after passive antibody administration in murine models of lethal bacteremia and endotoxemia has been observed (G.Nagy, unpublished). A possible action of the antibodies could be opsonization of bacteria to enhance uptake and killing by monocytes or capture free systemic LPS and prevent the engagement of TLR4 (data not shown).[116] In line with this, a murine O1 O-antigen-binding antibody has been shown to protect mice in a bacteremia model.[117] However, this antibody has never been followed up, probably due to only partial protection achieved at even high doses of at least 800 µg per mouse. Additionally, shedding of the O-antigen epitope due to the expressed K-antigens on the bacteria, could influence a possible protective effect of these antibodies.[17] Taken together, the identified antibodies could have a beneficial effect in preventing or treating *Kp* infections in humans, when passively administered.

6. Outlook

The data revealed that humans naturally acquire affinity-matured anti-glycan antibodies that cross-bind to different members of the microbiota. However, important questions remain which should be addressed by future studies. To get a first indication how this O-antigen specific memory is generated, the comparison of O-antigen-binding memory B cell repertoires in longitudinal studies from early infant to adults could reveal if these antibodies are efficiently elicited and sustained or need years of constant exposure to be generated.

In line with this, the identification of particular Ig genes, which are enriched in specific anti-O-antigen antibodies, could reveal a future prediction tool for a successful B cell memory response after vaccination. This suggests the generation of large sets of O-antigen-binding, mature naïve, and memory Ig repertoires of the same individual. This could potentially reveal particularly enriched Ig genes or Ig gene features present in the O-antigen-binding B cell population. This knowledge could provide a tool to either predict or control successful vaccination against *Kp* O-antigens.

Further, antibody reactivity measurement of O-antigen-binding B cells from *Kp* mono-colonized mice could reveal where these responses are generated and which cell types are involved in their generation. The knowledge gained from these experiments could broaden the understanding of how antibodies against glycan antigens of commensal bacteria are generated by the intestinal humoral immune system.

In addition, it needs to be clarified which functional effect cross-binding anti-glycan antibodies have on the microbiome. Thus, administration of O-antigen-specific IgA antibodies to mice combined with whole microbiota 16S rDNA sequencing could reveal changes in their microbial composition after exposure to O-antigen-specific antibodies. In line with this, sorting and cultivation of antibody-bound bacteria from human stool samples together with whole genome sequencing will reveal the additional species that are targeted by these antibodies and if they are beneficial or even pathogenic to the host. This could identify further potential bacterial targets of these antibodies.

Last, further experiments to test the protective capacity of O-antigen-specific antibodies in animal models of bacteremia, endotoxemia and pneumonia could reveal a beneficial effect of the antibodies in clearing the bacteria from the host or preventing septic shock. This could justify testing if passive administration of monoclonal antibody in acutely *Kp* infected human individuals results in a beneficial clinical outcome. In line with this, it could also be tested as prophylaxis for people at risk of infection, and therefore, could prevent the spread or infection by *Kp*.

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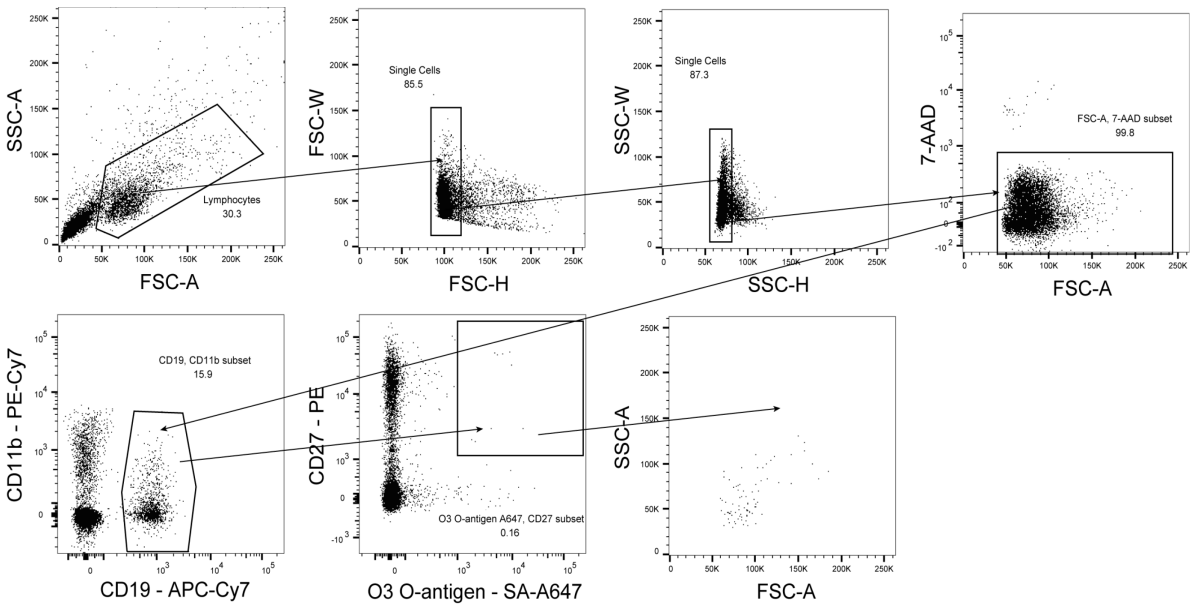
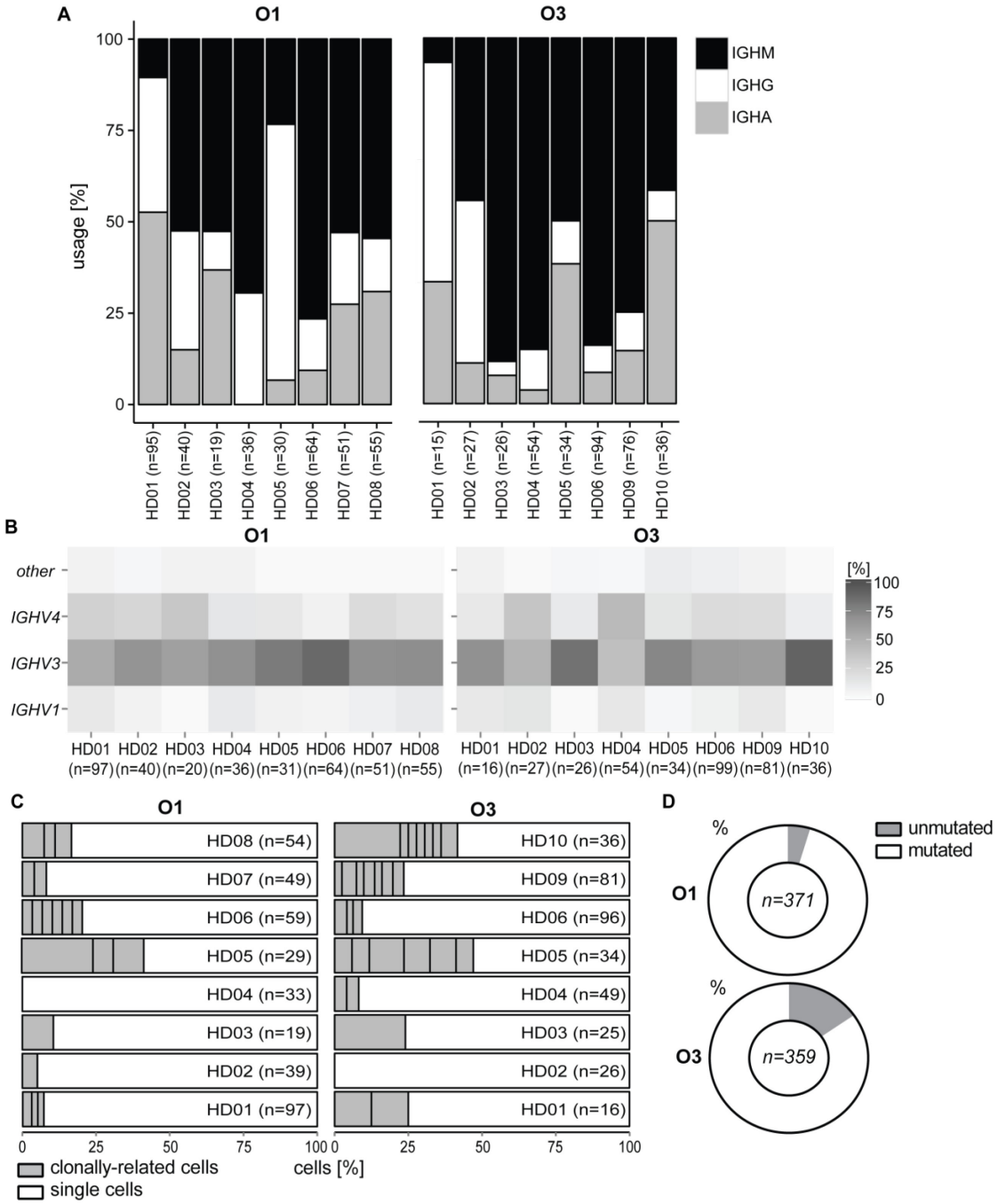
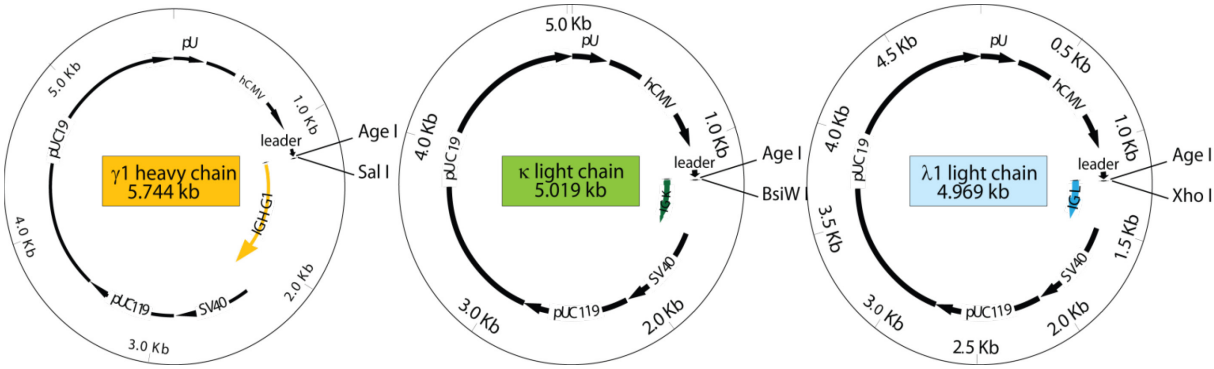
8. Supplementary Figures



Supplementary Figure 1: Representative gating strategy for the isolation of O3 O-antigen-binding memory B cells from peripheral blood of HD06



Supplementary Figure 2: Percentage of (A) Ig isotype, (B) IGHV gene family, (C) B cell cluster from O1 (left) and O3 (right) O-antigen-binding memory B cells per donor. (D) Percentage of Ig genes showing no somatic hypermutation in their IGHV gene pooled from all donors.



Supplementary Figure 3: Human Igγ1, Igκ and Igλ expression vectors containing the human Igγ1, Igκ and Igλ constant regions downstream of a multiple cloning site. A murine leader sequence is located upstream of the multiple cloning site. The whole peptide is under control of the human cytomegalovirus (hCMV) promotor. Used restriction sites are indicated and positive clones can be screened by resistance to ampicillin. Figure is adapted from Tiller. [118]

9. Supplementary tables

Antibody name	Hcluster	IGHV	IGHD	IGHJ	HCDR3	HSHM	Hconstant	IGKV	IGKJ	KCDR3	KSHM	IGLV	IGLJ	LCDR3	LSHM	O2+	O2-	ELISA	SA	Immunoblot	specific	Comment
KcPBO1007		IGHV3-53*01	IGHD6-19*01	IGHJ4*02	ATIKTYSSGWVPFDY	17	IGHM	IGKV1-5*03	IGKJ1*01	QQYNTYPWT	11					0	0	0	0	0	0	
KcPBO1010		IGHV4-38-2*01	IGHD4-17*01	IGHJ4*02	ARDRYGDPFDY	2	IGHM	IGKV3-20*01	IGKJ2*01	QQYGSSPYT	1					0	0	0	0	0	0	
KdPBO1011		IGHV4-39*01	IGHD1-26*01	IGHJ3*02	ARQEHEGAGACGYYGDDTFDI	12	IGHA2	IGKV3-11*01	IGKJ5*01	QQRSKWPPIT	2	IGLV8-61*01	IGLJ2*01			0	0	1	0	1	0	Excluded from analysis; probably binds K2 K-antigen
KcPBO1025	38	IGHV3-48*02	IGHD2-8*02	IGHJ4*02	AREKYCTVTDCLHGGSYFNS	13	IGHM					IGLV2-8*01	IGLJ1*01	NSYAGSNYV	7	0	0	1	0	1	1	
KdPBO1029		IGHV1-18*01	IGHD7-27*01	IGHJ2*01	ARKLGNWYFDL	19	IGHM	IGKV1-39*01	IGKJ4*01	QQSYSTPLT	0	IGLV2-23*02	IGLJ2*01	CSFSNSDTFVV	4	0	0	0	0	0	0	
KcPBO1031		IGHV3-33*01	IGHD6-19*01	IGHJ4*02	ARVSGAVARKNYFDY	0	IGHM	IGKV2D-30*01	IGKJ2*01			IGLV2-11*01	IGLJ1*01	CSYAGSYTLDYV	0	0	0	0	0	0	0	
KePBO1039		IGHV4-59*01	IGHD6-13*01	IGHJ3*02	ARQRASWSGVDI	27	IGHA1	IGKV3-20*01	IGKJ2*02	QQYGSSPWT	14					0	0	0	0	0	0	
KePBO1057		IGHV1-46*01	IGHD3-3*01	IGHJ1*01	AREYRDRDVIFHH	21	IGHA2	IGKV3-11*01	IGKJ5*01	QQRLNWPPIT	9					0	0	0	0	0	0	
KePBO1084	18	IGHV3-23*03	IGHD5-12*01	IGHJ4*02	AKDEGGSNLDYLDN	24	IGHA1	IGKV3-15*01	IGKJ2*01	QQYHNWPPYT	11					0	0	1	0	1	1	
KcPBO1088		IGHV3-23*01	IGHD5-5*01	IGHJ6*02	GAGGFHYGMDV	19	IGHM	IGKV3-20*01	IGKJ2*01	QQYTTSPYT	11					0	0	0	0	0	0	
KePBO1093		IGHV3-23*03		IGHJ4*02	ATDPGGQWLNYFDN	17	IGHA2	IGKV3-15*01	IGKJ2*01	QQYHNWPPYT	15					0	0	0	0	1	1	
KdPBO1101		IGHV1-18*01	IGHD3-9*01	IGHJ6*03	ARTYDFYYYYMDV	0	IGHM	IGKV3-15*01	IGKJ2*01	HQYNNWPHT	3					0	0	0	0	0	0	
KcPBO1103		IGHV3-53*01	IGHD6-19*01	IGHJ4*02	ARVVVTHSGWIPYDY	32	IGHA2	IGKV1-5*03	IGKJ1*01	QQYDTYPWT	9					0	0	0	0	1	0	Excluded from analysis; probably binds lipid A
KcPBO1106		IGHV3-23*01	IGHD2-21*01	IGHJ4*02	AKSDCGDGGCKLLDY	23	IGHM	IGKV1-5*03	IGKJ1*01	QQYNNDFPT	15					0	1	0	0	0	0	
KePBO1108	14	IGHV3-23*01		IGHJ4*02	MSGHVAN	23	IGHM	IGKV2-30*02	IGKJ1*01	MQGAHWPWA	9					0	0	1	0	1	1	
KePBO1111		IGHV4-31*03	IGHD4-17*01	IGHJ4*02	ARRSVTSGYFDY	10	IGHM	IGKV1-5*03	IGKJ2*01	QQYKSYPYT	5					0	0	0	0	0	0	
KePBO1117		IGHV1-2*02		IGHJ4*02	ARMRGVQGPR	10	IGHM					IGLV1-44*01	IGLJ2*01	AAWDGSLNSVV	5	1	1	0	0	0	0	
KcPBO1127		IGHV3-7*03	IGHD3-3*01	IGHJ3*02	ATDGGYWKFDI	19	IGHM					IGLV2-14*01	IGLJ3*02	SLYGSGRV	15	0	0	0	0	0	0	
KcPBO1142	42	IGHV3-49*04	IGHD6-6*01	IGHJ6*03	TRSAQIQGAGYYMDV	8	IGHA1	IGKV4-1*01	IGKJ2*01	HQYYSFPYT	3					0	0	0	0	0	0	
KePBO1144	18	IGHV3-23*03	IGHD5-12*01	IGHJ4*02	ARDEGGSNLDYLDN	20	IGHA2	IGKV3-15*01	IGKJ2*01	QQYHNWPPYT	12					0	0	1	0	1	1	
KePBO1147		IGHV3-23*01	IGHD7-27*01	IGHJ6*02	AKRMLPWGFMDV	15	IGHM	IGKV3-11*01	IGKJ4*01	QQRSKWPLT	5					0	0	0	0	0	0	
KdPBO1149		IGHV3-53*01		IGHJ4*02	ARGFSYFDY	4	IGHM	IGKV2-29*03	IGKJ1*01	MQGTHGKA	7	IGLV2-18*02	IGLJ2*01	SSYTSSSTLV	2	0	0	0	0	0	0	
KcPBO1151	90	IGHV3-30-3*01	IGHD3-3*01	IGHJ6*02	AITPASRYLYYYGLDV	13	IGHG2	IGKV2D-29*01	IGKJ5*01	MQSIQLPLT	3					0	0	0	0	0	0	
KcPBO1172		IGHV1-2*02	IGHD6-13*01	IGHJ6*01	ARDKRGAAGPYQGGPYYYYGMDV	17	IGHM	IGKV1-9*01	IGKJ2*01	QQLNYPHTS	10					0	0	0	0	0	0	
KdPBO1176		IGHV3-30-3*01	IGHD4-17*01	IGHJ4*02	AREGKATVTMTFDY	6	IGHG1	IGKV4-1*01	IGKJ1*01	QQYYISPRT	4					0	0	0	0	0	0	

KdPBO1182		IGHV3-23*01	IGHD6-19*01	IGHJ4*02	AGGSGWYYY	0	IGHM	IGKV1-12*01	IGKJ1*01	QQANSFPWT	0					0	0	0	0	0	0	
KdPBO1191		IGHV3-64*05	IGHD2-8*02	IGHJ4*02	VNRYGTGNANFDY	4	IGHM	IGKV4-1*01	IGKJ1*01	QQYYTTLGT	3					0	0	0	0	0	0	
KcPBO1196		IGHV3-15*04	IGHD3-16*01	IGHJ4*02	ATDAQWGR	19	IGHM	IGKV2-29*03	IGKJ1*01	MQDPWT	14					0	0	1	0	1	1	
KcPBO1202		IGHV3-7*02	IGHD3-16*01	IGHJ4*02	ARLMGDSTIWDY	25	IGHM	IGKV1-9*01	IGKJ5*01	QHRNSYPIT	8					0	0	0	0	0	0	
KePBO1207		IGHV3-23*01	IGHD2-2*02	IGHJ1*01	AKDPPYCISVGCGIPYKYSQH	11	IGHA1	IGKV4-1*01	IGKJ1*01	QQYYGTPWT	10					0	0	0	0	0	0	
KcPBO1208		IGHV3-53*01	IGHD6-13*01	IGHJ4*02	ARDAAAAATGTWG	5	IGHG2	IGKV3D-15*01	IGKJ5*01	QQYDNQWPT	3					0	0	0	0	0	0	
KePBO1213		IGHV3-23*01	IGHD6-19*01	IGHJ3*02	ARRYVSGWSTDGFGI	14	IGHM	IGKV3-11*01	IGKJ4*01	QQRNNWPLT	6					0	0	0	0	0	0	
KdPBO1221		IGHV3-13*01	IGHD6-6*01	IGHJ3*02	AREGAVVEAREYSSSSGAFDI	2	IGHM	IGKV1-33*01	IGKJ2*01	QQYDNLPPYT	0					0	0	0	0	0	0	
KdPBO1224	84	IGHV4-61*01	IGHD3-22*01	IGHJ6*03	VRDFIRRGCDSTHCRSMDV	23	IGHG2	IGKV3-15*01	IGKJ4*01	QQYNDWPLT	9					0	0	0	0	0	0	
KcPBO1229		IGHV3-72*01	IGHD2-8*01	IGHJ6*01	ARENCIGYCTYPPYYYYYAMDV	13	IGHM	IGKV1-39*01	IGKJ1*01	QQSYNTPRT	4					0	0	0	0	0	0	
KdPBO1233		IGHV3-23*01	IGHD2-15*01	IGHJ4*02	AKSDCGSGGCKLLNY	16	IGHA2	IGKV1-5*03	IGKJ1*01	QQYHNDSPT	15					0	1	0	0	0	0	
KcPBO1235		IGHV3-30*01	IGHD4-17*01	IGHJ4*02	ATTVTTTLGDY	3	IGHM	IGKV1-39*01	IGKJ1*01	QQSYSTPPWT	4					0	0	0	0	0	0	
KcPBO1343		IGHV3-48*03		IGHJ4*02	TRDKFDL	25	IGHM	IGKV2-30*01	IGKJ1*01	MQGTHWPWG	4					0	0	0	0	0	0	
KdPBO1347		IGHV3-23*01	IGHD2-21*02	IGHJ6*01	AKDRCGSNCYYRMDV	18	IGHM	IGKV2-28*01	IGKJ4*01	MQPLQTPLT	3					0	0	0	0	0	0	
KePBO1363		IGHV3-30-3*02	IGHD4-17*01	IGHJ5*02	AKDGPSRFSSTVTTGWFDP	10	IGHG2	IGKV1-39*01	IGKJ4*01	QQSHSVPFT	11					0	0	0	0	0	0	

Supplementary Table 1: Ig gene and antibody reactivity information from O1 O-antigen-binding B cells from peripheral blood

Antibody name	Hcluster	IGHV	IGHD	IGHJ	HCDR3	HSHM	Hconstant	IGKV	IGKJ	KCDR3	KSHM	IGLV	IGLJ	LCDR3	LSHM	ELISA	SA	INS	Immunoblot	specific	O3a	O3b	O5	*Sc* cell wall	Comment
KaPBO3-028		IGHV3-72*01	IGHD5-24*01	IGHJ4*02	VRVSHAYNYDY	18	IGHM	IGKV1-16*01	IGKJ4*01	QQYTSYPLT	8					0	0	0		0	n.d.	n.d.	n.d.	0	
KaPBO3-030		IGHV3-30*18		IGHJ4*02	AKDSITPFLYNVVSVPPAPADY	12	IGHM	IGKV3-20*01	IGKJ1*01	HHYGNSPGRA	13					1	1	1		0	n.d.	n.d.	n.d.	0	
KaPBO3-035		IGHV4-4*02	IGHD5-12*01	IGHJ4*02	VRGCEMVATNYYFDY	24	IGHA1	IGKV1-5*01	IGKJ3*01	QQYDTYPFT	16					1	1	0		0	n.d.	n.d.	n.d.	0	
KaPBO3-036	36	IGHV3-48*04		IGHJ4*02	VRGYLHNSFDF	16	IGHM	IGKV3-20*01	IGKJ1*01	QQYRSSLWT	5					1	0	0	1	1	1	0	0	0	
KaPBO3-045	83	IGHV4-61*08	IGHD2-8*02	IGHJ4*01	GGDCCTHVRWDY	40	IGHM					IGLV1-51*01	IGLJ3*01	AAWESSLAIVA	22	1	0	0	1	1	1	1	0	0	
KaPBO3-047	83	IGHV4-61*01	IGHD2-8*01	IGHJ4*02	AGDCCTNIRWDY	32	IGHM					IGLV1-51*01	IGLJ3*01	GVWESSLAIVA	12	1	0	0	1	1	1	1	0	0	
KbPBO3-055		IGHV4-b*01	IGHD2-15*01	IGHJ4*02	ARYCSGGSCPSRAFDY	2	IGHM	IGKV2-28*01	IGKJ2*01	MQALQTPYT	0					0	0	0	smear	0	n.d.	n.d.	n.d.	0	
KbPBO3-056		IGHV4-34*01	IGHD3-10*01	IGHJ1*01	ARVSPGTYYKYFQY	20	IGHG3	IGKV3-15*01	IGKJ4*02	QQYNNWPPLT	11					1	1	1		0	n.d.	n.d.	n.d.	0	
KbPBO3-059		IGHV3-11*05	IGHD5-12*01	IGHJ4*02	ARGRRPSQYIGYDK	11	IGHM	IGKV2-28*01	IGKJ1*01	MQALQTPHT	3					0	0	0		0	n.d.	n.d.	n.d.	0	
KbPBO3-060		IGHV1-18*01	IGHD4-4*01	IGHJ5*02	ARGSGYSDYYLS	11	IGHM	IGKV3-20*01	IGKJ1*01	QQYGSSPRT	0					0	0	0		0	n.d.	n.d.	n.d.	0	
KbPBO3-061		IGHV5-51*01	IGHD2-15*01	IGHJ4*02	ARQFRDCSGGSCYSGWFDP	0	IGHM	IGKV3-20*01	IGKJ1*01	QQYGSSPRT	0					0	0	0		0	n.d.	n.d.	n.d.	0	
KaPBO3-073		IGHV3-30*03	IGHD2-15*01	IGHJ4*02	GRDDEVVVVKAANSLVY	26	IGHA1	IGKV1-9*01	IGKJ2*01	QQLNSRPYT	9					1	0	0	1	1	1	0	0	0	
KaPBO3-074	36	IGHV3-48*04	IGHD1-1*01	IGHJ4*02	VRGYLHNSFDF	27	IGHA1	IGKV3-20*01	IGKJ1*01	QQYRFSLWT	9					1	0	0	1	1	1	0	0	0	
KaPBO3-075		IGHV3-11*01		IGHJ3*02	ARWNYAFDI	25	IGHA2	IGKV3-20*01	IGKJ1*01	QRYGDSPWT	12					0	0	0		0	n.d.	n.d.	n.d.	0	
KaPBO3-080	83	IGHV4-61*01	IGHD2-8*01	IGHJ4*02	AGDCCTHIRWDY	33	IGHM					IGLV1-51*01	IGLJ3*01	AAWESSLAIVA	23	1	1	1	1 & smear	0	1	1	1	1	shows some polyreactive properties
KaPBO3-081		IGHV4-4*02	IGHD3-10*01	IGHJ5*02	ASHITMVQGLISWFDP	5	IGHM	IGKV1-5*01	IGKJ1*01	QQYNRYPWT	5					0	0	0		0	n.d.	n.d.	n.d.	0	
KaPBO3-093		IGHV3-23*04	IGHD6-19*01	IGHJ4*02	TTHRSGPGWYF	21	IGHM					IGLV4-69*01	IGLJ3*01	QIWGTDFQLV	9	1	1	1		0	n.d.	n.d.	n.d.	0	
KaPBO3-096	37	IGHV3-48*04	IGHD6-19*01	IGHJ4*02	AAGTMAGGFNF	24	IGHM					IGLV1-51*01	IGLJ1*01	GAWDGSLSVYV	10	1	1	1	smear	0	1	0	0	0	
KbPBO3-100		IGHV1-18*01	IGHD3-16*02	IGHJ4*02	ARGDRSFSFFFDY	4	IGHM					IGLV1-51*01	IGLJ3*01	GAWDSSLSAVV	2	0	0	0		0	0	0	0	0	
KbPBO3-104	46	IGHV3-53*01	IGHD1-26*01	IGHJ4*02	ARDTHSGSRSDY	15	IGHM					IGLV1-44*01	IGLJ2*01	AAWDDSLNGVV	7	0	0	0	1	1	1	1	0	0	
KbPBO3-105		IGHV3-21*01	IGHD2-15*01	IGHJ5*02	ASCSGGSCLGGWFDP	6	IGHM	IGKV1-9*01	IGKJ2*01	QQLNSYPYT	1					0	0	0		0	0	0	0	0	
KbPBO3-113		IGHV4-b*01	IGHD4-4*01	IGHJ4*02	ASDQSNYRFDW	16	IGHA2	IGKV3-20*01	IGKJ4*01	QQYGSSPPLT	7					1	0	0	1	1	1	1	0	0	
KbPBO3-114		IGHV3-23*04	IGHD4-4*01	IGHJ4*02	AKAMMTGVVITTSFDY	14	IGHM	IGKV3-20*01	IGKJ4*01	HQYGSSPS	4					0	0	0		0	n.d.	n.d.	n.d.	0	
KbPBO3-115	47	IGHV3-53*01	IGHD1-26*01	IGHJ5*02	GADSHSGRRSDH	25	IGHM					IGLV1-44*01	IGLJ2*01	ATWDDRLNGVV	11	1	0	0	1	1	1	1	0	0	
KbPBO3-118		IGHV3-72*01	IGHD3-9*01	IGHJ4*02	ARVNYDSGHYNIAY	5	IGHM	IGKV1-16*02	IGKJ4*01	QHYKSYPLT	3					0	0	0		0	n.d.	n.d.	n.d.	0	
KaPBO3-127		IGHV1-8*01	IGHD1-7*01	IGHJ4*02	ARGGTIYGSRKEIDY	20	IGHG2	IGKV4-1*01	IGKJ1*01	QQYYSLPRT	9					0	0	0		0	0	0	0	0	
KaPBO3-139		IGHV4-59*01		IGHJ5*02	ARGALYKFDP	50	IGHA1					IGLV2-11*01	IGLJ3*01	CSKAGSYSS	4	1	1	1	smear	0	0	0	0	1	
KaPBO3-142		IGHV4-39*01	IGHD6-19*01	IGHJ1*01	ARPRGTAGEAIALFGFQH	6	IGHM	IGKV3-15*01	IGKJ1*01	QQYNNWPT	0					0	0	0		0	n.d.	n.d.	n.d.	0	

KaPBO3-144	37	IGHV3-48*04	IGHD6-19*01	IGHJ4*02	ATGTMAGGFDY	17	IGHM					IGLV1-51*01	IGLJ1*01	GTWDSSLSTYV	4	1	0	0	1	1	1	0	0	0	
KbPBO3-242		IGHV3-72*01	IGHD1-1*01	IGHJ4*02	ARPRGTNWNDLYFDY	3	IGHM	IGKV1-16*02	IGKJ4*01	QQYNSYPLT	1					0	0	0		0	n.d.	n.d.	n.d.	0	
KaPBO3-266		IGHV3-23*04		IGHJ4*02	AKKSGGPHPFDY	25	IGHG2	IGKV3-15*01	IGKJ2*01	QQYDNWPMYT	7					0	0	0		0	n.d.	n.d.	n.d.	0	
KaPBO3-278		IGHV3-9*01	IGHD3-22*01	IGHJ4*02	VKDMTMTANRLKGDFDY	7	IGHA2	IGKV3-11*01	IGKJ3*01	QQRYDWRT	4					1	1	0		0	0	0	0	0	
KaPBO3-286	24	IGHV3-30*04	IGHD6-19*01	IGHJ4*02	TREGYSSGRAPAFDY	26	IGHA2	IGKV1-5*03	IGKJ1*01	QRYDDYPVT	12					1	0	0	1	1	1	1	1	0	
KbPBO3-290	55	IGHV3-7*02	IGHD5-12*01	IGHJ4*02	WRGHYDRH	28	IGHA1	IGKV1-27*01	IGKJ1*01	QKYNSAPWT	8					0	0	0		0	0	0	0	0	
KbPBO3-291		IGHV4-61*08	IGHD6-13*01	IGHJ4*02	ARTGIATVGRHFDY	10	IGHG2	IGKV3-15*01	IGKJ4*01	QQYNNWPPLT	4					0	0	0		0	n.d.	n.d.	n.d.	0	
KbPBO3-298		IGHV3-23*04	IGHD1-26*01	IGHJ6*03	AKGFSREPYYSHMDV	15	IGHA1	IGKV1-39*01	IGKJ1*01	QQSYGTPLT	11	IGLV1-47*01	IGLJ3*02		0	0	0	0		0	n.d.	n.d.	n.d.	0	
KbPBO3-302	55	IGHV3-7*02	IGHD5-12*01	IGHJ4*02	WRGHYDRH	28	IGHA1	IGKV1-27*01	IGKJ1*01	QKYNSAPWT	8					0	0	0		0	-	-	-	0	
KbPBO3-339		IGHV4-4*02	IGHD4-17*01	IGHJ3*02	VKLQERYGAAFEI	21	IGHG1	IGKV1-39*01	IGKJ1*01	QQSYSTPRT	10					0	0	0		0	n.d.	n.d.	n.d.	0	
KaPBO3-368		IGHV4-31*03	IGHD6-6*01	IGHJ6*02	ATKMRVSRSSEGGETYYYYGIDV	17	IGHA1	IGKV1-8*01	IGKJ1*01	QQYYSYPRT	0	IGLV1-51*01	IGLJ3*01	GAWDSSMSAQE	7	0	0	1		0	n.d.	n.d.	n.d.	0	
KaPBO3-376		IGHV3-30*04	IGHD5-12*01	IGHJ4*02	ARDHGRYTGSYGLPGY	23	IGHG2	IGKV1-33*01	IGKJ3*01	QQFDNLPPFT	12					0	0	0		0	n.d.	n.d.	n.d.	0	

Supplementary Table 2: Ig gene and antibody reactivity information from O3 O-antigen-binding B cells from peripheral blood

Antibody name	Hcluster	IGHV	IGHD	IGHJ	HCDR3	HSHM	Hconstant	IGKV	IGKJ	KCDR3	KSHM	IGLV	IGLJ	LCDR3	LSHM	ELISA	SA	Immunoblot	O3a	O3b	O5	Sc cell wall
UaLPLO3008	1	IGHV3-23*01	IGHD2-8*01	IGHJ4*02	TKILNGRFDF	28	IGHA1	IGKV3-20*01	IGKJ2*02	QYYSASPRYA	34	IGLV1-40*02	IGLJ2*01	QSYDNSLSGSNV	10	0	0	0	0	0	0	0
UaLPLO3009	8	IGHV3-30*01	IGHD3-10*01	IGHJ4*02	AREGYSSGGCGAFDF	27	IGHA1	IGKV1-5*03	IGKJ2*01	QQYNSYPAT	10					1	0	1	1	1	0	0
UaLPLO3014	6	IGHV3-7*03	IGHD3-10*01	IGHJ4*02	ARGPNYGSRCDYLDF	22	IGHA1	IGKV2-28*01	IGKJ2*01	MQPLQTPYT	8					1	0	1	1	1	0	0
UaLPLO3025	5	IGHV3-64*05	IGHD3-9*01	IGHJ4*02	VKDAHTSDWEGG	24	IGHA1	IGKV2-30*01	IGKJ1*01	MQGVHWPRA	17					1	0	1	1	1	0	0
UaLPLO3029	5	IGHV3-64*05	IGHD3-9*01	IGHJ4*02	VKDAHTSDWEGG	25	IGHA1	IGKV2-30*01	IGKJ1*01	MQGVHWPRA	15					1	0	1	1	1	0	0
UaLPLO3062	5	IGHV3-64*05	IGHD3-9*01	IGHJ4*02	VKDAHTADWDGG	25	IGHA1	IGKV2-30*01	IGKJ1*01	MQGVHWPRA	19					1	0	1	1	1	0	0
UaLPLO3095	3	IGHV3-23*01	IGHD3-22*01	IGHJ4*02	AREGYSSGRCGSFDH	30	IGHA2	IGKV1-5*03	IGKJ1*01	QQYNDYSPA	11					1	0	1	1	1	1	0
UaLPLO3103	5	IGHV3-64*05	IGHD3-9*01	IGHJ4*02	VKDAHTADWEGG	30	IGHA1	IGKV2-30*01	IGKJ1*01	FQGTRWPRA	22					1	0	1	1	1	0	0
UaLPLO3105	6	IGHV3-7*03	IGHD2-21*02	IGHJ4*02	ARGPSYGDRCDFLDY	23	IGHA1	IGKV2-28*01	IGKJ2*01	MQTLEAPYT	19					1	0	1	1	1	0	0
UaLPLO3111	5	IGHV3-64*05	IGHD3-9*01	IGHJ4*02	VKDAHTADWDGG	27	IGHA1	IGKV2-30*01	IGKJ1*01	MQGVHWPRA	21					1	0	1	1	1	0	0
UaLPLO3113		IGHV3-23*01	IGHD2-15*01	IGHJ4*02	AREGYGSGHCGAFDY	27	IGHA2	IGKV1-5*03	IGKJ1*01	QQYNSYSPA	15					0	0	0	0	0	0	0
UaLPLO3131		IGHV4-4*02	IGHD5-24*01	IGHJ4*02	ARDLRDGYNWGIDY	16	IGHA1	IGKV3-11*01	IGKJ5*01	QQRFNWPPIT	12					0	0	0	0	0	0	0
UaLPLO3142	5	IGHV3-64*05	IGHD3-9*01	IGHJ4*02	VKDAHTSDWEGG	25	IGHA1	IGKV2-30*01	IGKJ1*01	MQGVHWPRA	18					1	0	1	1	0	0	0
UaLPLO3147	5	IGHV3-64*05	IGHD3-9*01	IGHJ4*02	VKDAHTSDWEGG	23	IGHA1	IGKV2-30*01	IGKJ1*01	MQGVHWPRA	18					1	0	1	1	0	0	0
UaLPLO3153		IGHV3-48*02	IGHD2-8*01	IGHJ4*02	AVSIEWYLHY	27	IGHA2	IGKV3-11*01	IGKJ1*01	QQRSKWPLT	20					1	0	1	1	1	0	0
UaLPLO3163		IGHV3-23*01		IGHJ4*01	PPDARLGELFLN	37	IGHA1	IGKV2-28*01	IGKJ1*01	MQARQTPWT	6					0	0	1	1	0	0	0
UaLPLO3171	1	IGHV3-23*01	IGHD2-8*01	IGHJ4*02	TKILNGRFDN	24	IGHA2	IGKV3-20*01	IGKJ2*01	QYYSGSPRYA	29					1	0	1	1	1	0	0
UaLPLO3175	5	IGHV3-64*05	IGHD3-9*01	IGHJ4*02	VKDAHTADWDGG	27	IGHA1	IGKV2-30*01	IGKJ1*01	MQGVHWPRA	20					0	0	1	1	0	0	0
UaLPLO3177	5	IGHV3-64*05	IGHD3-9*01	IGHJ4*02	VKDAHTGDWEGG	35	IGHA1	IGKV2-30*01	IGKJ1*01	MQGVHWPRA	13					0	0	1	1	0	0	0
UaLPLO3182		IGHV3-30*03	IGHD1-7*01	IGHJ2*01	ETGIRAPTTGPNWFFDL	29	IGHA1	IGKV1-5*03	IGKJ2*02	QEYTGQSF	14					1	1	smear	1	0	0	0
UaLPLO3183		IGHV3-23*01	IGHD3-9*01	IGHJ4*02	AKDGVSGNSVFDYFDF	17	IGHA2	IGKV3-20*01	IGKJ2*01	QQYGRSHSMYT	5					0	0	0	0	0	0	0
UaLPLO3191		IGHV3-30*01	IGHD3-22*01	IGHJ4*02	AREGHSSGHAPCFDY	30	IGHA2	IGKV1-5*03	IGKJ1*01	QQYNNYSPT	13					1	0	1	1	1	1	0
UaLPLO3208		IGHV4-39*05		IGHJ4*02	AWDSGSVERFDH	18	IGHA2					IGLV1-44*01	IGLJ2*01	AAWDDNFNGLL	16	1	0	1	1	1	1	1
UaLPLO3212	5	IGHV3-64*05	IGHD3-9*01	IGHJ4*02	VKDAHTGDWEGG	26	IGHA1	IGKV2-30*01	IGKJ1*01	MQGVHWPRA	17					1	0	1	1	1	0	0
UaLPLO3461	3	IGHV3-23*01	IGHD6-19*01	IGHJ4*02	AREGYSSGLCGCFDH	31	IGHA1	IGKV1-5*03	IGKJ1*01	QQYNSYPPW	13					1	0	1	1	0	1	0

UaLPLO3533	2	IGHV3-23*02	IGHD7-27*01	IGHJ3*02	AKDPNWAWGI	27	IGHA2	IGKV2-30*01	IGKJ1*01	MQGTYWPGT	24	IGLV1-51*01	IGLJ3*02	ATWDSSLSAGV	14	0	0	0	0	0	0	0
UaLPLO3537	3	IGHV3-23*01	IGHD6-19*01	IGHJ4*02	AREGYSSGLCGCFDY	20	IGHA1	IGKV1-5*03	IGKJ1*01	QQYNTYSLA	20					1	0	1	1	0	1	0
UaLPLO3547	9	IGHV3-7*03	IGHD4-17*01	IGHJ1*01	ARGPSYGDRSDYLDN	24	IGHA1	IGKV2-28*01	IGKJ2*01	MQPLKLPYT	13					1	0	1	1	1	0	0
UaLPLO3552	6	IGHV3-7*03	IGHD3-16*01	IGHJ4*02	ARGPSYGARTDFLDY	21	IGHA1	IGKV2-28*01	IGKJ2*01	MQPLQTPYT	16					1	0	1	1	1	0	0
UaLPLO3555	9	IGHV3-7*03	IGHD4-17*01	IGHJ1*01	ARGPSYGDRSDYLDN	24	IGHA1	IGKV2-28*01	IGKJ2*01	MQPLKLPYT	12					1	0	1	1	1	0	0
UaLPLO3583		IGHV3-23*01	IGHD3-10*02	IGHJ4*02	AKDYVVYNDGMWDFDF	44	IGHA1	IGKV1-39*01	IGKJ1*01	QESYTPLK	19					0	0	1	1	1	0	0
UaLPLO3598		IGHV1-69*13	IGHD3-10*01	IGHJ3*01	ARDTHEGLFSGPV	34	IGHA1	IGKV2-24*01	IGKJ1*01	MQATQYPRT	1					0	0	0	0	0	0	0
UaLPLO3601	6	IGHV3-7*03	IGHD2-21*02	IGHJ4*02	ARGPSYGDRCDYLDH	26	IGHA1	IGKV2-28*01	IGKJ2*01	MQPLQTPYT	15					1	0	1	1	1	0	0
UaLPLO3657	5	IGHV3-64*05	IGHD7-27*01	IGHJ4*02	VKDAHTADWDGG	28	IGHA1	IGKV2-30*01	IGKJ1*01	MQGVHWPRA	23					0	0	1	1	0	0	0
UaLPLO3666	2	IGHV3-23*01	IGHD7-27*01	IGHJ3*01	ARDPNWAWGV	30	IGHA2	IGKV2-30*02	IGKJ1*01	MQGTFWPGT	14					1	0	1	1	1	1	0
UaLPLO3671		IGHV3-30*10		IGHJ4*02	ARDIYSSGTVGTPDY	19	IGHA1	IGKV1-17*01	IGKJ3*01	LHHNTYPLT	10					0	0	0	0	0	0	0
UaLPLO3694		IGHV3-23*02		IGHJ3*02	AKDPNWPSGI	36	IGHA1	IGKV2-30*01	IGKJ1*01	LQGTYWPAT	23					1	0	1	1	1	1	0
UaLPLO3702		IGHV3-30*06	IGHD6-19*01	IGHJ4*02	AREVGSSGRCGFFDD	33	IGHA2	IGKV1-5*03	IGKJ1*01	QQYDSYPAT	12					1	0	1	1	1	0	0
UaLPLO3703	2	IGHV3-23*02	IGHD7-27*01	IGHJ3*02	AKDPNWAWGI	24	IGHA2	IGKV2-30*01	IGKJ1*01	MQGTFWPGT	19					0	0	0	0	1	1	0
UaLPLO3715		IGHV3-7*03	IGHD3-16*01	IGHJ4*02	ARGPHYGARCDYLDS	26	n.d.	IGKV1-39*01	IGKJ1*01	QQSYSIPWT	14					0	0	smear	0	0	0	0

Supplementary Table 3: Ig gene and antibody reactivity information from O3 O-antigen-binding B cells from lamina propria

donor	age	sex	sample	immune status	HIV status
HD01	57	m	peripheral blood	Sigma diverticulosis	n.d.
HD02	64	f	peripheral blood, Colon	Colon carcinoma	n.d.
HD03	61	f	peripheral blood	Suspected to colon carcinoma	n.d.
HD04	60	f	peripheral blood	Colon carcinoma	n.d.
HD05	63	m	peripheral blood, Colon	HNPCC colon carcinoma	n.d.
HD06	27	m	peripheral blood	healthy	Sero-negative
HD07	26	m	peripheral blood	healthy	n.d.
HD08	25	f	peripheral blood	healthy	n.d.
HD09	28	f	peripheral blood	healthy	n.d.
HD10	58	f	peripheral blood, term. Ileum	Suspected to Crohns disease	Sero-negative

Supplementary Table 4: Human donor information ; n.d. = not determined.

***IGH* primary PCR**	
Primer A	**Sequence**
Forward (Complete mix)	
5'L-VH1	ACAGGTGCCCACTCCCAGGTGCAG
5'L-VH3	AAGGTGTCCAGTGTGARGTGCAG
5'L-VH4/6	CCCAGATGGGTCCTGTCCCAGGTGCAG
5'L-VH5	CAAGGAGTCTGTTCCGAGGTGCAG
VH3 Leader-A	TAAAAGGTGTCCAGTGT
IGHV1,7-X1	ATGGACTGGACCTGGAG
IGHV1-X1-041	TCCTCTTTGTGGTGGCAGCAGC
IGHV2-X1-036	TCCACGCTCCTGCTRCTGAC
RM-IGHV4-X1	ATGAAACACCTGTGGTTCTTCC
Primer B	**Sequence**
Reverse	
hCh-mu-outer	GGAAGGAAGTCCTGTGCGAGGC
3' CgCH1	GGAAGGTGTGCACGCCGCTGGTC
hCh-al-outer	TGGGAAGTTTCTGGCGGTCACG

***IGH* secondary PCR**	
Primer C	**Sequence**
Forward	

RMX2-A	AGGTGCAGCTGCTGGAGTCKGG
Primer D	**Sequence**
Reverse	
3' CmCH1	GGGAATTCTCACAGGAGACGA
hCh-al-inner	GTCCGCTTTCGCTCCAGGTCACACT
IgG-internal	GTTCGGGGAAGTAGTCCTTGAC

***IGK* primary PCR**	
Primer A	**Sequence**
Forward	
5' L-Vk1/2	ATGAGGSTCCCYGCTCAGCTGCTGG
5' L-Vk3	CTCTTCCTCCTGCTACTCTGGCTCCCAG
5' L-Vk4	ATTTCTCTGTTGCTCTGGATCTCTG
Primer B	**Sequence**
Reverse	
3' Ck543	GTTTCTCGTAGTCTGCTTTGCTCA

***IGK* secondary PCR**	
Primer C	**Sequence**
Forward	
5' Pan-Vk	ATGACCCAGWCTCCABYCWCCCTG
Primer D	**Sequence**
Reverse	
3' Ck494	GTGCTGTCCTTGCTGTCCTGCT

***IGL* primary PCR**	
Primer A	**Sequence**
Forward	
5' L-Vl1	GGTCCTGGGCCCAGTCTGTGCTG
5' L-Vl2	GGTCCTGGGCCCAGTCTGCCCTG
5' L-Vl3	GCTCTGTGACCTCCTATGAGCTG
5' L-Vl4/5	GGTCTCTCTCSCAGCYTGTGCTG
5' L-Vl6	GTTCTTGGGCCAATTTTATGCTG
5' L-Vl7	GGTCCAATTCYCAGGCTGTGGTG
5' L-Vl8	GAGTGGATTCTCAGACTGTGGTG
Primer B	**Sequence**
Reverse	

hCl-057	CACCAGTGTGGCCTTGTTGGCTTG

***IGL* secondary PCR**	
Primer C	**Sequence**
Forward	
RM-IGLV-A	CAGYCTGYSCTGACTCA
RM-IGLV-B	TCCTATGAGCTGACWCAG
Primer D	**Sequence**
Reverse	
hCl-040	TCAGAGGAGGGYGGGAACAGAGTG

Supplementary Table 5: Ig gene primers used for amplification of IgHeavy and IgLight genes from human single B cells. Table was adapted from Murugan *et al.* [77]

IGHV primer		
Primer name	**Sequence [5' – 3']**	**IGHV segments**
5' AgeI VH1	CTGCAACCGGTGTACATTCCCAGGTGCAGCTGGTGCAG	IGHV1-2, IGHV1-46, IGHV1-69, IGHV1-8
5' AgeI VH1-18	CTGCAACCGGTGTACATTCCCAGGTTCAGCTGGTGCAG	IGHV1-3, IGHV1-18
5' AgeI VH1-24	CTGCAACCGGTGTACATTCCCAGGTCCAGCTGGTACAG	IGHV1-24, IGHV7-4-1, IGHV7-81
5' AgeI	CTGCAACCGGTGTACATTCCGAGGTGCAGCTGGTGCAG	IGHV5-51, IGHV5-a

VH1/5		
5' AgeI VH3-9	CTGCAACCGGTGTACATTCTGAAGTGCAGCTGGTGGAG	IGHV3-13, IGHV3-15, IGHV3-16, IGHV3-20, IGHV3-21, IGHV3-35, IGHV3-38, IGHV3-43, IGHV3-48, IGHV3-49, IGHV3-53, IGHV3-66, IGHV3-7, IGHV3-72, IGHV3-73, IGHV3-74, IGHV3-9, IGHV3-64 (except IGHV3-64*04)
5' AgeI VH3-23	CTGCAACCGGTGTACATTCTGAGGTGCAGCTGTTGGAG	IGHV3-23 (except IGHV3-23*04)
5' AgeI VH3-33	CTGCAACCGGTGTACATTCTCAGGTGCAGCTGGTGGAG	IGHV3-64*04, IGHV3-11, IGHV3-30, IGHV3-33
5' AgeI VH4	CTGCAACCGGTGTACATTCCCAGGTGCAGCTGCAGGAG	IGHV4-28, IGHV4-30-4, IGHV4-31, IGHV4-38, IGHV4-4, IGHV4-59, IGHV4-61, IGHV4-b
5' AgeI VH4-34	CTGCAACCGGTGTACATTCCCAGGTGCAGCTACAGCAGTG	IGHV4-34
5' AgeI VH4-39	CTGCAACCGGTGTACATTCCCAGCTGCAGCTGCAGGAG	IGHV4-30-02, IGHV4-39
5' AgeI VH6-1	CTGCAACCGGTGTACATTCCCAGGTACAGCTGCAGCAG	IGHV6-1
IGHJ primer		
Primer name	**Sequence [5' – 3']**	**IGHJ segments**
3' SalI	TGCGAAGTCGACGCTGAGGAGACGGTGACCAG	IGHJ1, IGHJ2, IGHJ4,

JH1/2/4/5		IGHJ5
3' SalI JH3	TGCGAAGTCGACGCTGAAGAGACGGTGACCATTG	IGHJ3
3' SalI JH6	TGCGAAGTCGACGCTGAGGAGACGGTGACCGTG	IGHJ6
IGKV primer		
Primer name	**Sequence [5' – 3']**	**IGKV segments**
5' AgeI Vk1-5	CTGCAACCGGTGTACATTCTGACATCCAGATGACCCAGTC	IGKV1-12, IGKV1-16, IGKV1-17, IGKV1-27, IGKV1-33, IGKV1-39, IGKV1-5, IGKV1-NL1, IGKV1D-12, IGKV1D-16, IGKV1D-33, IGKV1D-39
5' AgeI Vk1-13	CTGCAACCGGTGTACATTCTGCCATCCAGTTGACCCAGTC	IGKV1-13
5' AgeI Vk1D-43	CTGCAACCGGTGTACATTGTGCCATCCGGATGACCCAGTC	IGKV1-8, IGKV1D-43
5' AgeI Vk1D-8	CTGCAACCGGTGTACATTCTGTCATCTGGATGACCCAGTC	IGKV1D-8
5' AgeI Vk2-24	CTGCAACCGGTGTACATGGGGATATTGTGATGACCCAGAC	IGKV2-24, IGKV2-29, IGKV2-40, IGKV2D-24, IGKV2D-29, IGKV2D-40
5' AgeI Vk2-28	CTGCAACCGGTGTACATGGGGATATTGTGATGACTCAGTC	IGKV2-28, IGKV2D-28, IGKV4-1
5' AgeI Vk2-30	CTGCAACCGGTGTACATGGGGATGTTGTGATGACTCAGTC	IGKV2-30, IGKV2D-30, IGKV6D-41
5' AgeI Vk3-11	TTGTGCTGCAACCGGTGTACATTCAGAAATTGTGTTGACACAGTC	IGKV3-11, IGKV3-20, IGKV3-NL1/2/3/4/5, IGKV3D-11, IGKV3D-20, IGKV6-21, IGKV6D-21
5' AgeI	CTGCAACCGGTGTACATTCAGAAATAGTGATGACGCAGTC	IGKV2D-26, IGKV3-

Vk3-15		15, IGKV3-7, IGKV3D-15, IGKV3D-7
IGKJ primer		
Primer name	**Sequence [5' – 3']**	**IGKJ segments**
3' BsiWI Jk1/4	GCCACCGTACGTTTGATYTCCACCTTGGTC	IGKJ1, IGHJ4
3' BsiWI Jk2	GCCACCGTACGTTTGATCTCCAGCTTGGTC	IGKJ2
3' BsiWI Jk3	GCCACCGTACGTTTGATATCCACTTTGGTC	IGKJ3
3' BsiWI Jk5	GCCACCGTACGTTTAATCTCCAGTCGTGTC	IGKJ5
IGLV primer		
Primer name	**Sequence [5' – 3']**	**IGLV segments**
5' AgeI Vl1	CTGCTACCGGTTCCTGGGCCCAGTCTGTGCTGACKCAG	Used as primer mix for specific IGLV segment amplification. Human IGLV segments are summarized in Pallarès et al.[119]
5' AgeI Vl2	CTGCTACCGGTTCCTGGGCCCAGTCTGCCCTGACTCAG	
5' AgeI Vl3	CTGCTACCGGTTCTGTGACCTCCTATGAGCTGACWCAG	
5' AgeI Vl4/5	CTGCTACCGGTTCTCTCTCSCAGCYTGTGCTGACTCA	
5' AgeI Vl6	CTGCTACCGGTTCTTGGGCCAATTTTATGCTGACTCAG	
5' AgeI Vl7/8	CTGCTACCGGTTCCAATTCYCAGRCTGTGGTGACYCAG	
IGLJ primer		
Primer name	**Sequence [5' – 3']**	**IGLJ segments**
hCl-040-XhoI	CTCCTCACTCGAGGGYGGGAACAGAGTG	IGLJ1, IGLJ2, IGLJ3, IGLJ4, IGLJ5, IGLJ6, IGLJ7

Supplementary Table 6: Ig gene primers used for segment specific amplification of IgHeavy and IgLight genes from human single B cells.

Sequence [5' – 3']	**Primer name**
GACTACHVGGGTATCTAATCC	16S_V3V4_reverse
CACGTCTACCTACGGGNGGCWGCAG	16S_V3V4_forward_01
AGCTAGTGCCTACGGGNGGCWGCAG	16S_V3V4_forward_02
ACTATCGCCCTACGGGNGGCWGCAG	16S_V3V4_forward_03
GCGTATCACCTACGGGNGGCWGCAG	16S_V3V4_forward_04
ACTCTCCACCTACGGGNGGCWGCAG	16S_V3V4_forward_05
CGTCCATTCCTACGGGNGGCWGCAG	16S_V3V4_forward_06
AGCCGTAACCTACGGGNGGCWGCAG	16S_V3V4_forward_07
GAGTAGAGCCTACGGGNGGCWGCAG	16S_V3V4_forward_08
ACGTCGTTCCTACGGGNGGCWGCAG	16S_V3V4_forward_09
GTCCTGTTCCTACGGGNGGCWGCAG	16S_V3V4_forward_10
AGAAGCCTCCTACGGGNGGCWGCAG	16S_V3V4_forward_11
GAAGATCCCCTACGGGNGGCWGCAG	16S_V3V4_forward_12
TAGCTGAGCCTACGGGNGGCWGCAG	16S_V3V4_forward_13
ACGTCCAACCTACGGGNGGCWGCAG	16S_V3V4_forward_14
CACACATCCCTACGGGNGGCWGCAG	16S_V3V4_forward_15
CGGATCAACCTACGGGNGGCWGCAG	16S_V3V4_forward_16
TCAGCCTTCCTACGGGNGGCWGCAG	16S_V3V4_forward_17
AAGGCTCTCCTACGGGNGGCWGCAG	16S_V3V4_forward_18
TGTTCCGTCCTACGGGNGGCWGCAG	16S_V3V4_forward_19
GGAATGTCCCTACGGGNGGCWGCAG	16S_V3V4_forward_20
CATCCAAGCCTACGGGNGGCWGCAG	16S_V3V4_forward_21
GTCAACAGCCTACGGGNGGCWGCAG	16S_V3V4_forward_22
TCGCTATCCCTACGGGNGGCWGCAG	16S_V3V4_forward_23
AGCCTATCCCTACGGGNGGCWGCAG	16S_V3V4_forward_24
TCGGATTCCCTACGGGNGGCWGCAG	16S_V3V4_forward_25
CGGAGTATCCTACGGGNGGCWGCAG	16S_V3V4_forward_26
GAACCTTCCCTACGGGNGGCWGCAG	16S_V3V4_forward_27
AGAGGATGCCTACGGGNGGCWGCAG	16S_V3V4_forward_28
ACGCTTCTCCTACGGGNGGCWGCAG	16S_V3V4_forward_29
CACAGGAACCTACGGGNGGCWGCAG	16S_V3V4_forward_30
ACGAATCCCCTACGGGNGGCWGCAG	16S_V3V4_forward_31
CCTTCCATCCTACGGGNGGCWGCAG	16S_V3V4_forward_32
ATGGCGATCCTACGGGNGGCWGCAG	16S_V3V4_forward_33
AACGCCTTCCTACGGGNGGCWGCAG	16S_V3V4_forward_34
GTAAGGTGCCTACGGGNGGCWGCAG	16S_V3V4_forward_35
TGTCGACTCCTACGGGNGGCWGCAG	16S_V3V4_forward_36
ACTCTGAGCCTACGGGNGGCWGCAG	16S_V3V4_forward_37
GATGGAGTCCTACGGGNGGCWGCAG	16S_V3V4_forward_38
CTAGCTCACCTACGGGNGGCWGCAG	16S_V3V4_forward_39
CTGTACCACCTACGGGNGGCWGCAG	16S_V3V4_forward_40
CCTGTCAACCTACGGGNGGCWGCAG	16S_V3V4_forward_41
GGTCGTATCCTACGGGNGGCWGCAG	16S_V3V4_forward_42

CGCTGATACCTACGGGNGGCWGCAG	16S_V3V4_forward_43
TAGCTTCCCCTACGGGNGGCWGCAG	16S_V3V4_forward_44
CAAGTCGTCCTACGGGNGGCWGCAG	16S_V3V4_forward_45
GTCTCATCCCTACGGGNGGCWGCAG	16S_V3V4_forward_46
ACCAAGCACCTACGGGNGGCWGCAG	16S_V3V4_forward_47
AGTCAGGTCCTACGGGNGGCWGCAG	16S_V3V4_forward_48
TATCGCGACCTACGGGNGGCWGCAG	16S_V3V4_forward_49
TAGCAGGACCTACGGGNGGCWGCAG	16S_V3V4_forward_50
AGAAGGACCCTACGGGNGGCWGCAG	16S_V3V4_forward_51
TGAGCTGTCCTACGGGNGGCWGCAG	16S_V3V4_forward_52
CAGAGTGACCTACGGGNGGCWGCAG	16S_V3V4_forward_53
AGGTTCCTCCTACGGGNGGCWGCAG	16S_V3V4_forward_54
AGACCTTGCCTACGGGNGGCWGCAG	16S_V3V4_forward_55
CTTCCTTCCCTACGGGNGGCWGCAG	16S_V3V4_forward_56
CAGGTTCACCTACGGGNGGCWGCAG	16S_V3V4_forward_57
ACTGGTGTCCTACGGGNGGCWGCAG	16S_V3V4_forward_58
GGATTCACCCTACGGGNGGCWGCAG	16S_V3V4_forward_59
CACGATTCCCTACGGGNGGCWGCAG	16S_V3V4_forward_60
AGACATGCCCTACGGGNGGCWGCAG	16S_V3V4_forward_61
GACACAGTCCTACGGGNGGCWGCAG	16S_V3V4_forward_62
CCAGTTGACCTACGGGNGGCWGCAG	16S_V3V4_forward_63
CATGGATCCCTACGGGNGGCWGCAG	16S_V3V4_forward_64

Supplementary Table 7: Primers used for barcoded amplification of 16S rDNA samples

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